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Targeted glioma stem cells depletion through pharmacological WNT inhibition as a novel therapy for malignant brain tumors

Dissertation

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Zusammenfassung

Das Glioblastom ist der am häufigsten vorkommende hirneigene Tumor. Sogenannte Gliomstammzellen (GSZ), eine Subpopulation der Glioblastomzellen, werden aufgrund ihrer Resistenz gegenüber konventionellen Therapien für das Auftreten von Tumorrezidiven nach Operation, Strahlen- und Chemotherapie verantwortlich gemacht. Aktuelle Forschungsansätze versuchen GSZ direkt anzugreifen, um die Therapieresistenz von Glioblastomen zu überwinden und die Prognose der Patienten zu verbessern. GSZ werden von verschiedenen Stammzellsignalwegen gesteuert, von denen einige auch pharmakologisch modifiziert werden können. Einer dieser Signalwege ist der Wnt Signalweg. In dieser Arbeit soll die pharmakologische Wnt Inhibierung charakterisiert und ihr Effekt auf GSZ untersucht werden, um so die Frage beantworten zu können, ob sie eine Therapieoption für Glioblastompatienten darstellt. Die Behandlung von Glioblastomzellen mit dem pharmakologischen Wnt Inhibitor LGK974 führte zu einer Reduktion der intrinsischen Wnt Aktivierung. Des Weiteren zeigten die behandelten Zellen eine reduzierte Viabilität, eine erhöhte Apoptoserate, sowie reduzierte Stammzelleigenschaften *in vitro*. Außerdem wurde LGK974 mit dem Chemotherapeutikum Temozolomid (TMZ) und Strahlentherapie, die *first-line* Therapien für Glioblastome, kombiniert. LGK974 zeigte synergistische Effekte mit beiden Behandlungen *in vitro*, was zu der Annahme führte, dass pharmakologische Wnt Inhibierung die Chemo- und Bestrahlungsresistenz von Glioblastomen reduzieren kann. Eine umfassende Transkriptionsanalyse (*microarray analysis*) von Zellen welche mit LGK974, TMZ oder einer Kombination behandelt wurden zeigte, dass differenzielle Expression des Enzyms Aldehyddehydrogenase 3A1 (ALDH3A1) eine mögliche Ursache für die reduzierte Therapieresistenz in LGK974 behandelten Zellen darstellt. Um zu untersuchen, ob ALDH3A1 eine Rolle bei der Resistenzentwicklung gegen Chemotherapeutika spielt, wurde das Enzym genetisch gehemmt. ALDH3A1 *knock-down* Zellen zeigten eine verminderte generelle Viabilität und eine erhöhte Sensitivität gegenüber TMZ Behandlung als die Kontrollzellen. Außerdem zeigten ALDH3A1 supprimierte Zellen eine reduzierte Expression diverser Stammzellmarker sowie eine verminderte Klonogenität, was auf eine Reduktion der GSZ Population hinweisen kann. Zusammenfassend zeigt diese Arbeit, dass die Behandlung mit dem Wnt Inhibitor LGK974 eine potentielle Therapie für Glioblastome darstellt, um GSZ direkt anzugreifen. Dies wird wahrscheinlich durch die Reduktion der ALDH3A1 Expression vermittelt. Um die aktuelle Datenlage zu pharmakologischer Wnt Inhibition in Glioblastomen darzustellen, wurden die bisherigen Daten durch ein *review* vervollständigt, das alle an Glioblastomen getesteten, publizierten Medikamente auflistet und diskutiert.

Abstract

Glioblastoma is the most common primary brain tumor. A subpopulation of glioblastoma cells defined as glioma stem-like cells (GSCs) are thought to be highly resistant against conventional therapies, leading to recurrence of the tumor after surgery and conventional chemo- and radiotherapy. New research approaches aim to directly target GSCs to overcome treatment resistance in glioblastoma and improve the prognosis of patients. GSC formation and maintenance is driven by several stem cell pathways, which can be targeted by pharmacological compounds. One of them is known as the Wingless (Wnt) pathway. This work aims to characterize the effects of pharmacological Wnt inhibition in glioblastoma, its effect on GSC maintenance, and possible application as a novel anti-GSC treatment strategy. Therefore, *in vitro* GSC models were treated with the pharmacological Wnt inhibitor LGK974, leading to a reduction of intrinsic Wnt activation. Additionally, treatment with LGK974 decreases cell viability, increases apoptosis and impairs stemness *in vitro.* Furthermore, LGK974 treatment was combined with either DNA alkylating agent temozolomide (TMZ) or γ-irradiation, which are the currently used standard-of-care treatment options for glioblastoma patients. Both treatments showed synergistic effects when combined with LGK974 *in vitro*, leading to the assumption that pharmacological Wnt inhibition can reduce chemo- and radioresistance of GSCs. In order to further elucidate the underlying link between Wnt signaling and therapy resistance, microarray analysis revealed reduced mRNA expression of the enzyme aldehyde dehydrogenase 3A1 (ALDH3A1) upon combined LGK974 and TMZ treatment. To further assess whether ALDH3A1 promotes chemoresistance in glioblastoma cells, its expression was suppressed. ALDH3A1 knock-down cells showed reduced cell viability in general, and more reduction of cell viability than control cells under TMZ treatment. Since ALDH3A1 expression correlates with stemness, we assessed the stemness geno- and phenotype of ALDH3A1 suppressed cultures and observed both reduced stem cell marker expression and clonogenicity. Taken together, this study highlights pharmacological Wnt suppression as a potential novel glioblastoma therapy directly targeting GSCs, and alleviating therapy resistance, plausibly through reduction of ALDH3A1 expression. To further evaluate the current status of pharmacological Wnt inhibition in glioblastoma, a review summarizing and discussing the published work about all compounds tested on glioblastoma samples completes this work.

Abbreviations

2-HG	2-hydroxygluatate
ABCB1	ATP binding cassette subfamily B member 1
abs	absolute
ALDH3A1	aldehyddehydrogenase 3A1
APC	adenomatous-polyposis-coli
ATRX	alpha-thalassemia/mental retardation syndrome X-linked
AXIN	axis inhibitor protein
BBB	blood brain barrier
CBP	CREB-binding protein
CD133	prominin1
CED	convection-enhanced delivery
CI	combination index
CSC	cancer stem-like cell
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DIPG	diffuse intrinsic pontine glioma
DKK	dickkopf
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
GAPDH	glyceraldehyde 3-phophate dehydrogenase
GBM	glioblastoma multiforme
GLI1/2	glioma-associated oncogene homolog 1/2
GSC	glioma stem-like cell

GSK-3β	glycogen synthase kinase 3β
EGFRvIII	epidermal growth factor receptor variant III
em	emission
EMT	epithelial-to-mesenchymal transition
EVI	evenness interrupted
ex	excitation
FC	fold change
FDA	U. S. Food and Drug Administration
Fzd	frizzled
GMT	glial-mesenchymal transition
GTP	guanosine-5'-triphosphate
IC_{50}	half maximal inhibitory concentration
IDH	isocitrate dehydrogenase
JNK	c-Jun N-terminal protein kinase
LEF	lymphoid enhancer factor
LGG	low-grade glioma
LRP	lipoprotein receptor-related protein
GFAP	glial fibrillary acidic protein
Gy	gray
MAP2	microtubule-associated protein 2
MB	medulloblastoma
MDR1	multidrug resistance protein 1
mg	milligram(s)
MGMT	O-6-methylguanine-DNA-methyltransferase

min	minute(s)
ml	milliliter(s)
mm	millimeter(s)
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
µm	micrometer(s)
µmol	micromole
n	amount
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitrotetrazolium blue chloride
NFAT	nuclear factor of activated T-cells
NF1	neurofibromin1
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NLK	nemo-like kinase
nm	nanometer(s)
no.	numero
p	probability value
PD-1	programmed cell death protein 1
PDGFR	plate-derived growth factor receptor
PI3K	phosphatidylinositol 3-kinase
PTEN	phosphatase and tensin homolog
Rac	Ras-related C3 botulinum toxin substrate
Raf	rapidly accelerated fibrosarcoma
Rho	Ras homologue

RIPA	radioimmunoprecipitation assay
RMA	robust multi-array average
RNA	ribonucleic acid
RNF43	ring finger protein 43
ROR2	tyrosine-protein kinase transmembrane receptor 2
Ryk	receptor-like tyrosine kinase
SD	standard deviation
sFRP	secreted frizzled protein
Shh	sonic hedgehog
SMO	smoothened
SNP	single nucleotide polymorphisms
STAT3	signal transducer and activator of transcription 3
SVZ	subventricular zone
TAA	tumor-associated antigens
TCF	T-cell specific transcription factor
TCGA	The Cancer Genome Atlas
TCIA	The Cancer Imaging Archive
TGFβ	transforming growth factor β
TMZ	temozolomide
TP53	tumor protein 53
TSA	tumor-specific antigens
TTF	tumor treating fields
VEGF	vascular endothelial growth factor
vs	versus

WHO	World Health Organization
WIF-1	Wnt inhibitory factor 1
WISP1	Wnt1 inducible signaling pathway protein 1
Wnt	wingless
ZEB1	zinc finger E-box-binding homeobox 1

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Introduction

Classification of gliomas

Gliomas are tumors that occur primarily in the brain and the spinal cord. The name “glioma” is based on the fact that these tumors resemble glia cells and was introduced by Rudolf Virchow in the 19th century, who described glia cells as “connective tissue” in the brain (1). In 1926, Harvey Cushing and Percival Bailey published the first classification of gliomas, distinguishing different subtypes according to the specific sort of glia cells they share the most morphologic features with, including astrocytoma, oligodendroglioma and ependymoma, resembling astrocytes, oligodendrocytes and ependymal cells (2). Over time, brain tumors were not exclusively classified by their morphology, but further by their grade of malignancy according to the World Health Organization (WHO). The first classification for tumors of the central nervous system was introduced in 1979 and revised four times since then. The WHO distinguishes between four different tumor grades, based on histological aspects. Grade I gliomas (e.g. pilocytic astrocytoma) are characterized by circumscribed growing patterns and lack of malignant progression. Patients can often be cured by surgery. Grade II to IV gliomas are nowadays regarded as a separate group, plausibly arising from a different cell of origin, since they are characterized by infiltrative growth pattern and a tension to progress into higher tumor grades by time. Grade II gliomas are marked by increased cellularity and nuclear atypia, grade III gliomas further present with higher mitotic activity. Grade IV gliomas are particularly called glioblastoma and characterized by tumor necrosis and microvascular proliferation additionally to grade III tumor’s criteria. Glioblastomas account for more than half of all diagnosed gliomas.

In 2016, the WHO published its latest classification, introducing for the first time molecular markers complementing the histological characteristics (3). The WHO classification now distinguishes between isocitrate dehydrogenase (IDH)1/2 wildtype and IDH1/2 mutant gliomas. This is a very early occurring mutation in tumor development causing malfunctional IDH1/2 proteins which cannot fulfill their designated functions as part of the citric acid cycle. Mutated IDH proteins

metabolize isocitrate into 2-hydroxygluatate (2-HG) instead of α-ketoglutarate. 2-HG accumulations further promote histone and DNA-methylation, leading to tumorigenesis. Interestingly, IDH1/2 mutations are generally found in low-grade gliomas and secondary glioblastomas, which are developed from former diffuse or anaplastic astrocytomas and tend to have a better prognosis. It is thought that IDH1/2 mutations lead to reduced capacity of nicotinamide adenine dinucleotide phosphate (NADPH) production in tumor cells, which makes them more vulnerable to oxygen species released due to chemo- and radiotherapy. Primary glioblastomas, formed de novo and more frequently diagnosed in elderly patients, are defined by an IDH wildtype genotype. Primary glioblastomas account for 90% of all diagnosed glioblastomas (4). Another important molecular marker for classification of gliomas are alterations on chromosome arms 1p and 19q. 1p/19q co-deletion is highly associated with the oligodendrocytic phenotype and according to the latest WHO classification required to diagnose a tumor as an "oligodendroglioma". 1p/19q co-deletion is also associated with higher response to chemotherapy and prolonged survival (5–7). The astrocytic phenotype is linked to *tumor protein p53* (*TP53*) mutations and alterations of alpha-thalassemia/mental retardation syndrome X-linked (ATRX) expression (8,9). *TP53*, a well studies tumor suppressor gene, is inactivated due to loss of function mutations in approximately every second glioma, and represents an unfavorable prognostic factor (10). Loss of ATRX expression is linked to a better prognosis in anaplastic astrocytoma (11). Another tumor entity that often shows loss of ATRX expression is diffuse midline glioma (named diffuse intrinsic pontine glioma before 2016). Primarily occurring in the brain stem, thalamus, and spinal cord of children, diffuse midline gliomas are defined by mutations in the H3K27 protein. *H3K27* is a gene encoding for the histone variant H3.3. Loss of function has an impact on epigenetic processes in the tumor cell and may lead to genomic instability (12). Nevertheless, these processes are poorly understood so far.

A classification for glioblastoma based on genomic alterations was described by Verhaak et al. in 2010. Due to differential expression profiles of genes including the *Epidermal Growth Factor Receptor (EGFR)*, *Neurofibromin1 (NF1)*, *Plate-Derived Growth Factor Receptor A (PDGFRA),* and *IDH1* they defined four

different glioblastoma subgroups, termed classical, mesenchymal, proneural and neural subtype (13). The subtypes differ in response to treatment, showing best treatment response in classical glioblastoma and worst in proneural tumors.

Localization and origin of glioma

Regarding anatomic distribution, 40% of glioma cases occur in the frontal lobe, followed by 29% in the temporal lobe. 14% occur in the parietal lobe, whereas only 3% are localized in the occipital lobe. A correlation between localization and tumor grading due to WHO classification could not be shown in the past (14). Nevertheless, a direct correlation between the distribution of glioblastomas in relation to the subventricular zone (SVZ) and the molecular subgroups defined by Verhaak et al. could be identified. Based on clinical imaging, proneural and neural glioblastoma were described to be more likely localized close to the SVZ, in contrast to mesenchymal and classical glioblastoma, that occur predominantly far from the SVZ (15).

The SVZ is a structure of the brain located at the boarder walls of the lateral ventricles, where neural stem and glial progenitor cells reside. There, they have optimal conditions to proliferate and generate neurons and glia cells. It is hypothesized that gliomas arise from these multipotent cells in the SVZ. From there, mutated cells migrate into different parts of the brain and form infiltrative tumors. Driver mutations during the formation of low-grade gliomas are thought to be IDH1/2 mutations, since they occur very early in glioma development. Further genetic instability leads to either *TP53* mutations or deletion of 1p/19q determining differentiation into astrocytomas or oligodendrogliomas, respectively. Primary glioblastomas often show amplification of *EGFR*, which is thought to be a driver mutation for IDH1/2 wildtype gliomas (16,17).

Nevertheless, it remains elusive which external or internal factors influence the formation of glioblastoma. The only defined risk factors are hereditary familial syndromes such as neurofibromatosis (type 1 and 2), tuberous sclerosis, von Hippel-Lindau, or Li-Fraumeni-syndrome. The only described risk factor not involving hereditary genetic alterations is exposure to ionizing radiation,

especially to the head and neck, which could be correlated to glioblastoma occurrence. Incidence rates of glioblastoma rise with the age and could be correlated to the sex of the patients, with males being more often affected. Aberrations on chromosome 10 and 17 further increase the risk (18,19). In contrast to most other cancer types, smoking does not seem to increase the probability for developing glioblastoma. Despite rumors about radiofrequency radiation from mobile phones and microwave ovens promoting tumorigenesis, there is no reported correlation with occurrence of brain tumors.

Treatment of glioblastoma

Overall survival time of patients newly diagnosed with glioblastoma and treated with current therapeutic standards remains poor with 12 to 15 months on average (20). Without treatment, patients only survive a few months. Treatment of glioblastoma typically includes surgery followed by radiotherapy (60 Gy, 5 days per week in doses of 1.8 – 2 Gy) and adjuvant therapy with temozolomide (TMZ) (21), an orally administered chemotherapeutic drug. The alkylating agent TMZ adds alkyl or methyl groups to the DNA, mostly on the N-7 or O-6 positions of guanine residues. Expression of the O-6-methylguanine-DNA methyltransferase (*MGMT*) antagonizes the effect of alkylating drugs such as TMZ, making the tumor more resistant towards chemotherapy. Therefore, silencing of the *MGMT* gene due to promoter methylation, which often occurs in high-grade gliomas, is an indicator whether the tumor is sensitive or resistant towards chemotherapy with TMZ (22,23). During surgery, Carmustine-polymer wafers, also known as Gliadel, can be placed in the resection cavity to work as local chemotherapeutic (24). However, survival benefit compared to placebo is low (25).

Other treatment options include an antiangiogenic monoclonal antibody, bevacizumab (Avastin), targeting the vascular endothelial growth factor (VEGF) A. Unfortunately, after accelerated approval by the FDA for recurrent glioblastoma in 2009, expected therapeutic efficacy has not been that apparent, and latest studies indicate that bevacizumab treatment has no survival benefit for patients suffering from glioblastoma. One possible reason for the lack of

effect of bevacizumab and most other pharmacological compounds is the obstacle of passing the blood brain barrier (BBB) to reach sufficient concentrations at its target side. Convection-enhanced delivery (CED) via intracranial catheters which deliver substances directly to their side of action is one subject of current research. Nevertheless, a completed phase III study by Vogelbaum et. al on glioblastoma patients (using citredexin besudotox, a pseudomonas exotoxin with recombinant human interleukin-13 in the study group and Gliadel in the control group) could not show any benefit (26).

New therapeutic opportunities apart from traditional pharmaceuticals and radiation treatment include electric-field therapy developed by Novocure, so called tumor treating fields (TTF). The electronic fields are installed at the patient's scull. The theory is that TTFs only target dividing tumor cells while no harming non-proliferative healthy brain tissue. Initially approved for recurrent glioblastoma, the FDA expanded the initial approval for TTF as first-line therapy, after a study showed survival benefit for patients treated with a combination of TTF and TMZ compared to patients that only received TMZ (20.5 vs. 15.6 months, p=0.004) (27).

Immunotherapy is considered a standard of care for many malignancies. To date, there has been no approval for immunotherapy in glioblastoma. However, extensive research and several clinical studies are in progress, one of them focusing on T-cell based vaccines. In this approach the immune system shall be sensitized, evoking immune response against glioblastoma cells by injecting antigens which are exclusively present (tumor-specific antigens, TSA) or mostly present (tumor-associated antigens, TAA) in the tumor. The TSA epidermal growth factor variant III (EGFRvIII) was believed to be a potential target. Unfortunately, EGFRvIII vaccine failed to show a survival benefit in combination with TMZ, presumably due to low expression in heterogeneous glioblastoma tumor mass (28). Alternatively, autologous dendritic cells, initially activated with TAAs are reinjected into the brain. As antigen-presenting cells, these dendritic cells are supposed to constantly present the TAAs on their cell surface and thus induce a continuous tumor-specific T-cell response. An ongoing clinical phase III trial will prove efficacy (29). Exposing dendritic cells to TAA overexpressed in GSCs showed promising results in a phase II study (30). As for metastatic

melanoma, immune checkpoint inhibitors have emerged as a powerful tool (31). Blocking antibodies against the immunosuppressive receptors cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) are presumed to maintain T-cell activation and immune response. Several clinical trials testing checkpoint inhibitors on glioblastoma are ongoing (32,33).

Glioma stem cell therapies

In 2004, Singh et al. described cells within brain tumors which possess high self-renewal capacity and the ability to initiate tumor growth, so called tumor-initiating cells or cancer stem cells (CSCs) (34,35). CSCs were first discovered in acute myeloid leukemia in the late 1990s (36,37) and subsequently described also in solid malignant tumor entities such as breast (38), colon (39), head and neck (40), pancreas (41), lung (42), liver (43) and skin cancer (44). In glioma, CSCs are also known as glioma stem-like cells (GSCs). Markers enriched in neural stem and progenitor cells are used to differentiate between GSCs and non-GSC-brain tumor cells. Most prominent is the transmembrane glycoprotein and neural stem cell surface marker CD133, also known as prominin1 (35,45). Later on, GSCs were found to be highly resistant against radio- and chemotherapy, causing predictable treatment failure with common standard therapeutic regimes (46–49).

It is presumed that GSCs account for a small percentage of the tumor cells' population. Although conventional therapies result in tumor shrinkage since they target the bulk of the tumor, highly resistant GSCs are left behind and give rise to an even more aggressive tumor relapse (50). Therefore, new therapy approaches are needed, capable of precisely targeting GSCs. As a consequence, many studies aim to pharmaceutically inhibit so called CSC pathways. These signaling pathways are mostly well described in somatic stem cells, where they assist in maintaining an optimal niche for these cells. Several stem cell pathways were described to be aberrantly activated in GSCs.

One of them is the Notch pathway, which plays an essential role in embryonic development and inhibition of neuronal cell differentiation (51). In cancer, the

four different Notch receptors (named NOTCH1, 2, 3 and 4) can show aberrant function through mutations, making them an interesting target for CSC therapies (52). Gamma-secretase inhibitors prevent processing of membrane proteins like Notch receptors and are used experimentally as well as in clinical trials to block Notch signaling (53). It was shown that gamma-secretase inhibitors effectively deplete brain tumor stem-like cells in mice as well as in glioblastoma patients (54,55).

The Sonic hedgehog (Shh) pathway is also known for its importance in GSC maintenance and correlated to chemoresistance in glioblastoma (56,57). Three groups of Shh pathway inhibitors are used, operating on different target locations. The first group targets the membrane-bound protein smoothened (SMO), thereby impeding activation of the transcription factors glioma-associated oncogene homolog 1 and 2 (GLI1 and GLI2), leading to repressed transcription of several pro-tumorigenic genes. Several SMO inhibitors were tested in clinical trials in various types of cancers. Vismodegib and erismodegib were approved by the FDA for application in basal cell carcinoma. At present, a phase II clinical study is ongoing for treating recurrent glioblastoma with vismodegib (58). Another group of Shh inhibitors directly targets Gli transcription factors. Since GLI1 and GLI2 can also be activated by other molecular pathways, independently from SMO, Gli inhibitors are also effective in tumors harboring mutations of the Shh pathway. Clinical trials are ongoing for several types of cancer, not including brain tumors. Operating upstream of Shh signaling, the third group of Shh inhibitors consists of compounds targeting SHH, a hedgehog ligand (59). This group is still under preclinical development.

CSC pathways are often interconnected. For instance, a group from Boston describes a link between Shh and phosphatidylinositol 3-kinase (PI3K) signaling in glioblastoma (60). PI3K is frequently activated by loss of the tumor suppressor gene phosphatase and tensin homolog (PTEN). PI3K leads to AKT (protein kinase B) activation that further activates mTOR (mechanistic target of rapamycin). In dividing cells, PTEN is suppressed by the transforming growth factor (TGF)-β. In almost 60% of glioblastoma, the PI3K pathway harbors mutations (61). An ongoing phase II trial tests a PI3K inhibitor on recurrent glioblastoma with activated PI3K pathway (62). Another phase II study is using

the AKT inhibitor perifosine in recurrent glioblastoma, but little effect was shown so far (63). mTOR inhibitors are already well established in the clinic for treating e.g. colon cancer (64), breast cancer (65) and non-small cell lung cancer (66). Several clinical trials are ongoing to test the effectiveness of mTOR inhibitors on brain tumors, but so far none of the compounds got approved for glioblastoma (67).

Wnt signaling pathway

In the early 1980s, a new protooncogene called int1 (integration 1) was described in mice transfected with breast cancer virus. Simultaneously, another gene involved in embryonic tissue development was found in Drosophila and named Wingless. Later on, it was discovered that both genes were identical, and a common name was chosen: Wnt. Further research revealed that the protein encoded by the Wnt gene was integrated into a network of proteins interacting with each other in a signaling cascade with Wnt at its starting point. The newly discovered pathway was called Wnt signaling. Nowadays, we distinguish between several different Wnt proteins (labeled by numbers) and two different signaling cascades, the canonical and the noncanonical Wnt signaling pathway.

The canonical Wnt pathway was the first to be found and is much better understood. It is activated by the proteins Wnt-1, -2, -3, -8a, -8b, -10a, and -10b which bind to a receptor named Fizzled and its co-receptors low-density lipoprotein receptor-related protein (LRP)5 or LRP6. This leads to recruitment of axis inhibitor protein (Axin), a negative Wnt regulator, to the plasma membrane. During absent Wnt activation, Axin gathers together with glycogen synthase kinase 3β (GSK-3β) and adenomatous-polyposis-coli (APC) protein to form a destruction complex, leading to the degradation of β-catenin. When instead Wnt signaling is activated, the destruction complex is not able to be formed and β-catenin accumulates within the cell, enters the cell's nucleus, and binds as a co-activator to transcription factors from the TCF/LEF (transcription factor/ lymphoid enhancer factor) family. Thereby, it drives transcription of several target genes involved in cell growth and motility.

The noncanonical Wnt signaling pathway is less described and understood. Activation is induced by Wnt proteins 4, 5a, 5b, 6,7a, 7b, and 11. While activated, intracellular calcium is released, leading to increasing levels of nemo like kinase (NLK). NLK inhibits the β-catenin/TCF transcription complex, making noncanonical Wnt signaling a direct counterpart of canonical Wnt signaling. Calcium release further activates nuclear factor of activated T cells (NFAT). NFAT plays a pivotal role in immune response, in cancer it was shown to increase cell motility and metastasis formation (68). Independently of calcium release, the c-Jun N-terminal protein kinase (JNK) pathway is activated through the GTPases Rho (Ras homologue) and Rac (Ras-related C3 botulinum toxin substrate). There is evidence that JNK has an essential role in cell polarity, growth and differentiation.

Besides embryogenesis, Wnt signaling has a critical role in cancer development and maintenance. Many tumor entities are defined by mutations related to the Wnt pathway or epigenetically silenced Wnt antagonists. The second case is more applicable to glioblastoma (69). Wnt activation leads to transcription of genes essential for proliferation and migration of cancer cells, further leading to tumor growth and metastasis. One mechanism that is enrolled in metastatic cancer and induced by Wnt signaling is called epithelial-to-mesenchymal-transition (EMT). This genetic reprogramming originating from embryonic development makes tumor cells losing their cell-cell adhesions and transforming into a mesenchymal state with increased invasive potential. Wnt signaling and EMT were both linked to resistance against chemo- and radiotherapy. Genes encoding for multidrug transporter proteins such as *ATP binding cassette subfamily B member 1/ multidrug resistance protein 1* (*ABCB1/MDR1)* were found to be transcriptional target genes of canonical Wnt signaling (70). Furthermore, Wickström et al. showed that expression of the repair enzyme MGMT was regulated through Wnt signaling in brain tumors. In their work they could restore chemosensitivity among glioblastoma cell lines through Wnt suppression (71). Downstream targets of Wnt like *Wnt1 Inducible Signaling Pathway Protein 1* (*WISP1)* were found to be involved in maintaining resistance against γ-irradiation in cancer cells (72). In prostate cancer, Wnt activation was linked to expression of the oxidation enzyme aldehyde dehydrogenase (ALDH)

and radioresistance (73). Nevertheless, the underlying mechanisms initiating resistance mechanisms upon Wnt activation remain incompletely understood.

Pharmacological Wnt inhibition

As described previously, there are two different types of Wnt signaling pathways with antagonizing effects, which makes is difficult to modulate both at the same time. In this study LGK974 (Wnt-974) was used, a small molecule pan-Wnt inhibitor modulating both pathways and created by Novartis. It is a porcupine inhibitor, interfering with the palmitoylation of Wnt proteins which is necessary for entering the intercellular space and binding to its receptors. Thereby inhibitory effects are equal among all different Wnt proteins *in vitro*. LGK974 was also shown to be effective in a ring finger protein 43 (RNF43)-mutant pancreatic cancer, a head and neck squamous cell carcinoma and a breast cancer mouse model *in vivo* (74,75). The compound is currently tested in a clinical phase I study on solid tumors dependent on Wnt ligands, including pancreatic cancer, B-Raf (rapidly accelerated fibrosarcoma) mutant colorectal cancer, melanoma, triple negative breast cancer, head and neck cancer and other tumor types with documented upstream genetic alteration in Wnt signaling (76). Whereas LGK974 is not the only pharmacological compound interfering with Wnt signaling, most agents solely inhibit the canonical branch of the Wnt pathway. An overview of all compounds tested on glioblastoma is presented in the review "Clipping the Wings of Glioblastoma: Modulation of WNT as a Novel Therapeutic Strategy" included in this work.

Objective

Glioblastoma is a malignant brain tumor with a dismal prognosis, presumably caused by a highly resistant cell fraction within the tumor, so-called GSCs. Although pharmaceutical compounds trying to directly target stem cell pathways that are crucial for GSC maintenance have been tested, none of them has made it into clinic for glioblastoma so far. Much uncertainly still exists about the impact of Wnt signaling in glioblastoma. This work wants to further address this issue.

The main aim of this thesis is to investigate whether pharmacological Wnt inhibition can be used as a potential therapy in glioblastoma. The thesis includes three publications, each one covering a different subtopic.

The first paper examines whether the pharmacological Wnt inhibitor LGK974 is capable of targeting GSCs *in vitro*. To address this question a reporter assay construct containing seven TCF binding sides followed by a firefly luciferase cassette was used to test whether Wnt activation is suppressed after LGK974 treatment in three glioblastoma cell lines. Effects on cell growth, proliferation and cell death were examined as well as stemness characteristics including expression of stem cell markers (NANOG, CD133), differentiation markers (GFAP, MAP2) and clonogenic potential. To assess the role of Wnt activation in glioblastoma *in vivo*, 73 tumor samples from glioblastoma patients were stained for intranuclear β-catenin. Furthermore, data published by The Cancer Genome Atlas (TCGA) was analyzed to elucidate a possible correlation between the overall survival time of glioma patients and the Wnt activation status of the tumor (77).

The second publication intends to determine whether and how LGK974 can increase the tumors` sensitivity towards chemo- and radiotherapy in glioblastoma *in vitro*. Therefore, we analyzed whether a combination treatment of glioblastoma cell lines with LGK974 and either γ-radiation or TMZ has synergistic effects. In order to demonstrate a possible synergistic effect of LGK974 treatment and the MGMT methylation status, we used two cell lines with methylated and two with unmethylated MGMT promoter. To reveal which mechanism causes synergy of combined LGK974 and TMZ treatment, a

microarray assay was performed, highlighting ALDH3A1 to be significantly downregulated upon combined treatment. To further assess the role of ALDH3A1 in glioblastoma, TMZ sensitivity, clonogenicity and stem cell marker expression (CD133, Nestin, Sox2) were tested in ALDH3A1 knock-down cells (78).

The third paper is a review that gives an overview of all pharmacological Wnt inhibitors which were tested on glioblastoma at the time of publication. Different compounds are closely examined concerning their mechanism of action and possible applicability for glioblastoma treatment. Furthermore, the effects of canonical and noncanonical Wnt pathway activation are outlined in greater detail. Additionally, we have a closer look on Wnt signaling in glioblastoma listing common pathway alterations and evaluating markers for Wnt activation (79).

Taken together, this work combines experimental *in vitro* research on a pharmacological Wnt inhibitor in glioblastoma with a detailed overview of the current state of the art on pharmacological Wnt inhibition in gliomas. This work further describes the first application of a small-molecule Wnt inhibitor on glioblastoma cell lines, which has only been tested on pancreatic and head and neck cancer cell lines before. As therapies directly targeting GSCs in patients are missing so far, the results are highly relevant for new clinical approaches combating glioblastoma. Over the long term the results of this thesis can form a basis for *in vivo* experiments and clinical trials.

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ORIGINAL ARTICLE

Pharmacologic Wnt Inhibition Reduces Proliferation, Survival, and Clonogenicity of Glioblastoma Cells

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Abstract

Wingless (Wnt) signaling is an important pathway in gliomagenesis and in the growth of stem-like glioma cells. Using immunohistochemistry to assess the translocation of β-catenin protein, we identified intranuclear staining suggesting Wnt pathway activation in 8 of 43 surgical samples (19%) from adult patients with glioblastoma and in 9 of 30 surgical samples (30%) from pediatric patients with glioblastoma. Wnt activity, evidenced by nuclear β-catenin in our cohort and high expression of its target AXIN2 (axis inhibitor protein 2) in published glioma datasets, was associated with shorter patient survival, although this was not statistically significant. We determined the effects of the porcupine inhibitor LGK974 on 3 glioblastoma cell lines with elevated AXIN2 and found that it reduced Wnt pathway activity by 50% or more, as assessed by T-cell factor luciferase reporters. Wnt inhibition led to suppression of growth, proliferation in cultures, and modest induction of cell death. LGK974 reduced NANOG messenger RNA levels and the fraction of cells expressing the stem cell marker CD133 in neurosphere cultures, induced glial differentiation, and suppressed clonogenicity. These data indicate that LGK974 is a promising new agent that can inhibit the canonical Wnt pathway in vitro, slow tumor growth, and deplete stem-like clonogenic cells, thereby providing further support for targeting Wnt in patients with glioblastoma.

Key Words: AXIN2, β-Catenin, CD133, Glioma, LGK974, Wnt.

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INTRODUCTION

Patients with glioblastoma multiforme (GBM) rarely survive more than 2 years after the initial diagnosis, making this malignant glioma one of the most lethal tumors overall (1). Glioma stem cells (GSCs) are thought to play a key role in long-term tumor growth and resistance to standard therapies. This subpopulation of cells is defined by its capacity for indefinite self-renewal and by its ability to initiate orthotopic xenograft formation (2, 3). Glioma stem cells also show multilineage differentiation potential (3–5), express markers found in nonneoplastic neural stem cells (6–9), and are relatively resistant to radiation and chemotherapy compared with glioma cells lacking stem cell properties (10–12). Therefore, many research groups, including our own, have sought to identify molecular regulators required for the survival and proliferation of GSCs and to evaluate them as new therapeutic targets. One pathway that has been implicated in GSCs and glioma pathobiology is the wingless (Wnt) pathway (13–17).

The Wnt pathway has been shown to regulate a range of cellular interactions in normal development and diseases (18–20). It is a major pathway among stem and progenitor cells in the developing fetus (21, 22) and in adults (20, 23). For example, Pinto et al (24) modulated Wnt activity in the intestines of transgenic mice and showed that the pathway is required for stem cell homeostasis. Wnt is also required for maintenance of neural stem cells, and loss of signaling induces neural, glial, and oligodendroglial differentiation (25–27).

Wnts are a large family of highly conserved protein ligands that are modified by lipids and palmitate before they are secreted as paracrine factors (28) and before they bind to cell surface receptors of the Frizzled family and to their low density lipoprotein receptor-related protein (LRP) coreceptors (29). Ligand/receptor binding recruits the downstream mediator Disheveled to the receptor site (30). Cytoplasmic levels of the key factor β-catenin (CTNNB1) are regulated through a specific degradation complex composed of the scaffolding protein AXIN (axis inhibitor protein), a product of the tumor-suppressor gene *adenomatous polyposis coli*, casein kinase 1, and glycogen synthase kinase 3, which promote its phosphorylation and constitutive proteolytic degradation (18). Wnt binding and recruitment of Disheveled disrupt this inactivation complex and lead to accumulation of free CTNNB1 in the cytoplasm, which translocates into the nucleus, binds to transcriptional coactivators of the T-cell factor (TCF)/lymphoid enhancer factor (LEF) family, and

promotes expression of genes involved in a variety of cellular processes important in tumorigenesis, including growth (31–33), invasion (34–36), and therapeutic resistance (37, 38). One well-characterized Wnt target is AXIN2 (39–41). AXIN2 expression has previously been shown to directly correlate with Wnt activity and aggressive behavior in GBM model systems (42–44).

In gliomas, Wnt is generally activated at the level of ligand interaction rather than mutation (45). For example, the gene *EVI*, which is responsible for the secretion of Wnt morphogens, is frequently overexpressed in GBM (46). Moreover, increased expression of the pathway receptors Frizzled 2 and Frizzled 9 and of Wnt6 by the zinc finger protein PLAGL2 promotes tumor growth by impeding glioma differentiation (15). Wnt5a has been shown to promote invasion by inducing the extracellular matrix metalloproteinase MMP-2 (47) and the proliferation of GBM cells (48). Wnt3a increases nuclear translocation of CTNNB1 through induction of the cell cycle regulator FoxM1, thereby enhancing gliomagenesis (38, 44). In addition, high levels of the Wnt receptor Frizzled 4 augment glioma invasion and therapeutic drug resistance through induction of epithelial to mesenchymal transition and reduced susceptibly to inducing caspase3-dependent apoptosis (49).

A number of prior studies have used modulation of upstream activators or genetic methods to inhibit Wnt in gliomas. In general, these have shown inhibitory effects on GSCs and overall tumor growth (14, 36, 49, 50). However, direct pharmacologic suppression of Wnt activity in cancers has been challenging because of a lack of effective and specific Wnt inhibitors. Recently, potent inhibitors of the Wnt-specific acyltransferase porcupine, which lead to disruption of the ligand-driven activation of the pathway, have been developed; these hold considerable promise as potential

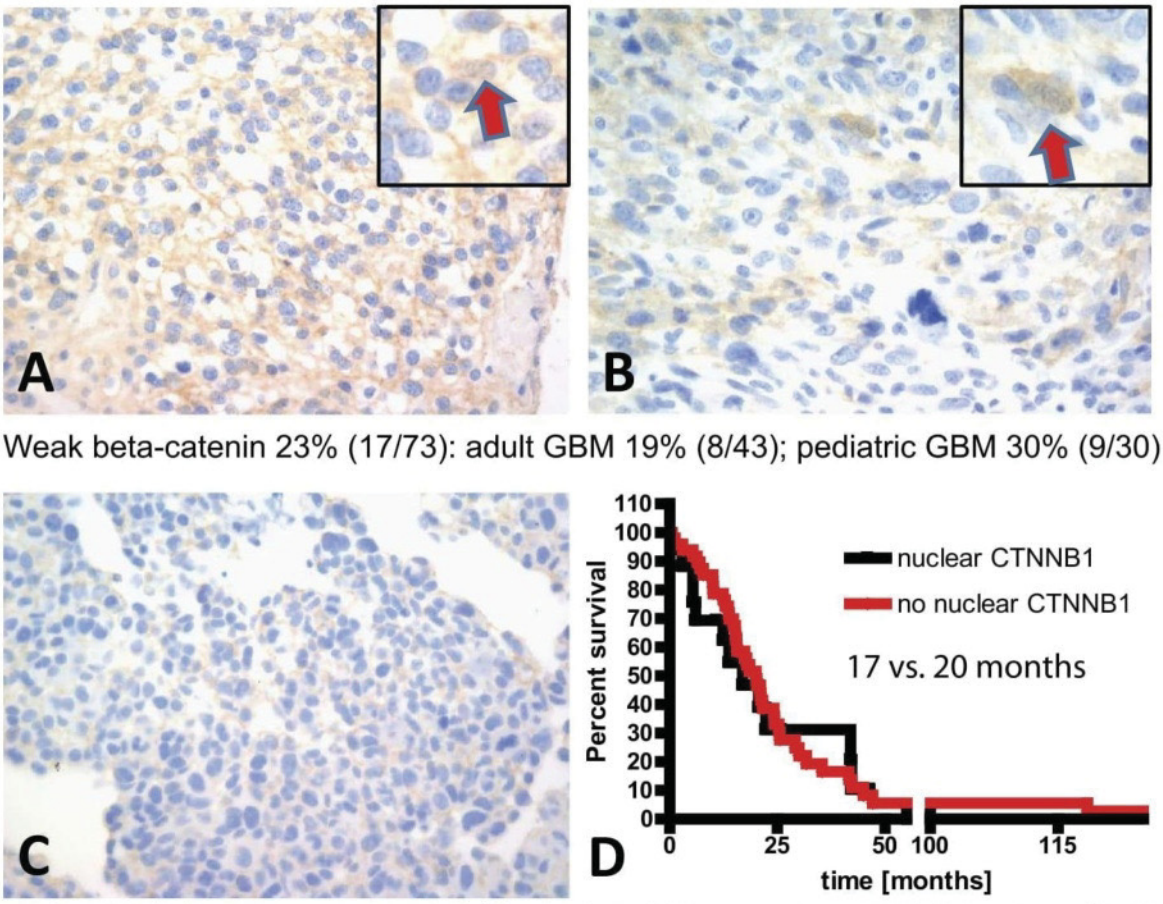


FIGURE 1. CTNNB1/β-catenin expression in surgical adult and pediatric glioblastoma specimens. **(A)** Glioblastoma in an adult with moderate cytoplasmic and weak nuclear β-catenin immunoreactivity in a subset of cells (inset; arrow). **(B)** Pediatric glioblastoma with weak β-catenin expression in cytoplasm and scattered nuclei (inset; arrow). **(C)** A weak cytoplasmic staining pattern with no nuclear protein was detected in formalin-fixed pellets from the GBM1 neurosphere cell line. **(D)** Patients whose glioblastoma contain nuclear β-catenin have shorter overall survival than those without signs of Wnt activity (median overall survival, 17 vs 20 months; $p = 0.8$).

treatment. One such agent, LGK974, has shown therapeutic potential in experimental studies of head and neck, breast, and pancreas cancers (51–53). An open-label Phase 1 clinical trial for various tumor types with documented genetic alterations upstream of the Wnt pathway (https://clinicaltrials.gov/ct2/show/NCT01351103) is currently further investigating the clinical effects of this compound.

In this study, we demonstrate the ability of LGK974 to inhibit canonical Wnt signaling in several in vitro GBM models with AXIN2 expression levels similar to those seen in primary tumor specimens. Glioblastoma multiforme cells treated with the Wnt inhibitor also showed significant reductions in overall cell growth, decreased proliferation and clonogenicity, lower expression of the stem cell marker CD133, and induction of glial differentiation. Taken together, these results suggest that targeting the Wnt pathway in GBM using the porcupine inhibitor LGK974 may represent a novel treatment strategy for malignant gliomas.

MATERIALS AND METHODS

Primary Tissue Samples, Cell Culture Models, and Pharmacologic Treatment

Snap-frozen samples from adult brain gliomas (GBM: p349, p635, p636, p696, and p770; low-grade glioma: p824) were retrieved from the Johns Hopkins Neuropathology Brain Tumor Tissue Bank. Tissue collection and analyses were approved by the Johns Hopkins Institutional Review Board. Neurosphere cell lines (GBM1, JHH520, U87NS, and GBM10) were maintained in serum-free Dulbecco modified Eagle medium/F12 (Life Technologies, Carlsbad, CA) supplemented with B27 (Life Technologies), bovine fibroblast growth factor (Peprotech, Rocky Hill, NJ), human epidermal growth factor (Peprotech), and heparin (Sigma-Aldrich, St Louis, MO), as previously described (54). The adherent glioma cell line LN229 was cultured in Dulbecco modified Eagle medium (Life Technologies) containing 10% fetal calf serum (Life Technologies) and 1× penicillin/streptomycin (Life Technologies). GBM1 was generously provided by A. Vescovi (Milan, Italy); AQH612 was provided by A. Quinones-Hinojosa (Department of Neurosurgery, Johns Hopkins Hospital, Baltimore, MD); and JHH520 and JHH136 were provided by G. Riggins (Johns Hopkins Hospital). LN229 and U87 were purchased from American Tissue Culture Collection (Manassas, VA). GBM10 (54) and GBM14 (55) are neurosphere lines generated in our laboratory from intraoperative specimens obtained from the Department of Neurosurgery at Johns Hopkins Hospital. RNA was extracted from cell lines and tumor samples using standard techniques. RNA from the pediatric brain tumor cell lines SF188, SU-DIPG, BT35, BT40, Res186, Res259, D283, and D425 were generously provided by E. Raabe (Department of Pediatric Oncology, Johns Hopkins Hospital.

Identities of cell cultures were confirmed by analyzing 9 tandem repeats plus a sex-determining marker, Amelogenin, using the StemElite kit (Promega, Madison, WI), at the John Hopkins Core Facility for DNA Fragment Analyses (http://grcf.med.jhu.edu/) as part of its standard short tandem repeat fingerprinting service for cell lines (Supporting Information File, Supplemental Digital Content 1, http://links.lww.com/NEN/A774). Cells were passaged before the porcupine inhibitor LGK974 (no. M60106-2S; Xcess Biosciences, San Diego, CA) was applied at the indicated concentration and dissolved in cell line–specific cell culture media. Cell cultures were supplied with LGK974 every 48 hours in fresh media.

A tissue microarray containing multiple 0.6-mm cores from 35 pediatric and 45 adult glioblastoma samples was constructed as previously described (56). The tissue array and GBM1 cell pellets, which were fixed in formalin, processed, and sectioned in the same manner as clinical specimens, were stained for CTNNB1/β-catenin (no. 610154, 1:1000 dilution; Transduction Laboratories, Lexington, KY) at the Johns Hopkins Hospital Clinical Pathology Laboratory. Tumors were scored by a neuropathologist (Charles G. Eberhart), who

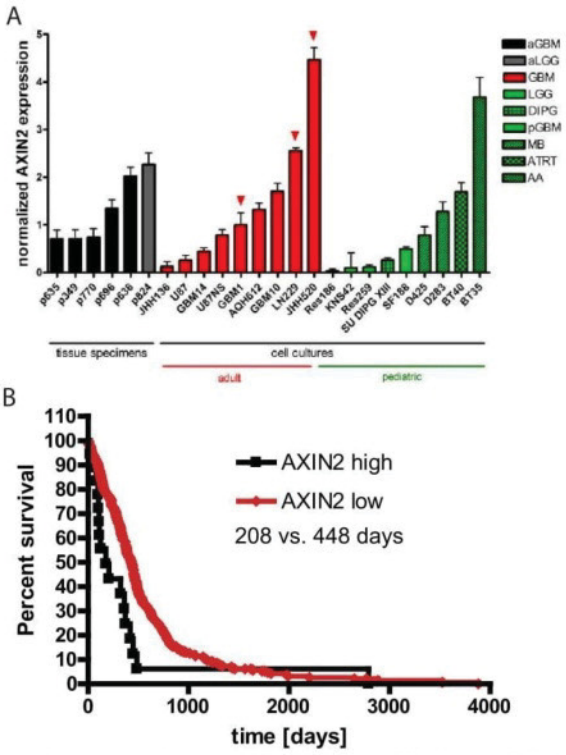


FIGURE 2. Quantification of the Wnt target AXIN2 and clinical prognosis. **(A)** AXIN2 mRNA levels in snap-frozen adult brain tumors (GBM: p349, p635, p636, p696, and p770; low-grade glioma [LGG]: p824), and brain tumor cell lines derived from adult GBM (GBM1, GBM10, GBM14, JHH136, JHH520, AQH612, U87, U87NS, and LN229) and pediatric brain tumors, including diffuse intrinsic pontine glioma (SU-DIPG), anaplastic astrocytoma (AA; BT35), atypical teratoid rhabdoid tumor (ATRT; BT40), LGG (Res186 and Res259), high-grade gliomas (SF188 and KNS42), and medulloblastoma (MB; D283 and D425). **(B)** Patients with GBM who have high AXIN2 have significantly shorter survival compared with patients with low AXIN2 (median overall survival, 208 vs 448 days) (*z*-score = +1.75, p = 0.06; TCGA dataset May 2015).

was blinded to clinical and pathologic findings, as having *no*, *weak*, *moderate*, or *strong* cytoplasmic/surface and nuclear immunoreactivity for CTNNB1 in at least 5% of cells.

Reporter Assay for Measurement of Wnt/CTNNB1 Activity

Canonical Wnt pathway activity in in vitro material was assessed using bioluminescence-based quantification with luciferase reporter construct (firefly luciferase cassette under the control of 7 TCF binding sites, as previously described (57). This reporter, which measures occupied CTNNB1 TCF/LEF binding sites, was stably integrated into cells. Infectious lentiviral particles carrying the reporter were generated using a third-generation lentiviral packaging system, as described previously (58, 59); stable integration was selected using 2 μg/mL puromycin (Sigma-Aldrich). Cells overexpressing Wnt because of the introduction of point-mutated CTNNB1/β-catenin served as positive controls and were generated in our laboratory as previously described (34).

For each measurement, cells were harvested, washed in 1× PBS, and lysed according to the manufacturer's instructions using the Dual-Light luciferase and β-galactosidase reporter gene assay system (no. T1003; Life Technologies). Luminescence readout was performed at 490 nm emission wavelength on an Infinite M1000Pro plate reader (Tecan, Morrisville, NC) and normalized to β-galactosidase activity.

Analysis of Gene and Protein Expression

Abundance of messenger RNA (mRNA) transcripts was assessed by conversion into complementary DNA and subsequent relative quantification using SYBR green–based fluorescence (Bio-Rad, Hercules, CA). Real-time polymerase chain reaction normalized to the housekeeping gene *β-actin* was performed with the $\Delta\Delta C_t$ method. Primer sequences can be found in Supporting Information File, Supplemental Digital Content 2 (http://links.lww.com/NEN/A775). Western blot analysis was performed as described previously (60), and antibodies were used following the manufacturer's instructions (Supporting Information File, Supplemental Digital Content 2, http://links.lww.com/NEN/A775).

For The Cancer Genome Atlas (TCGA) analyses, AXIN2 transcription levels acquired (using the Agilent 244k microarray) from 401 GBM specimens collected from TCGA (http://cancergenome.nih.gov) were retrieved through cBioPortal (http://www.cbioportal.org) (61) from the provisional Glioblastoma dataset in May 2015. AXIN2 expression values

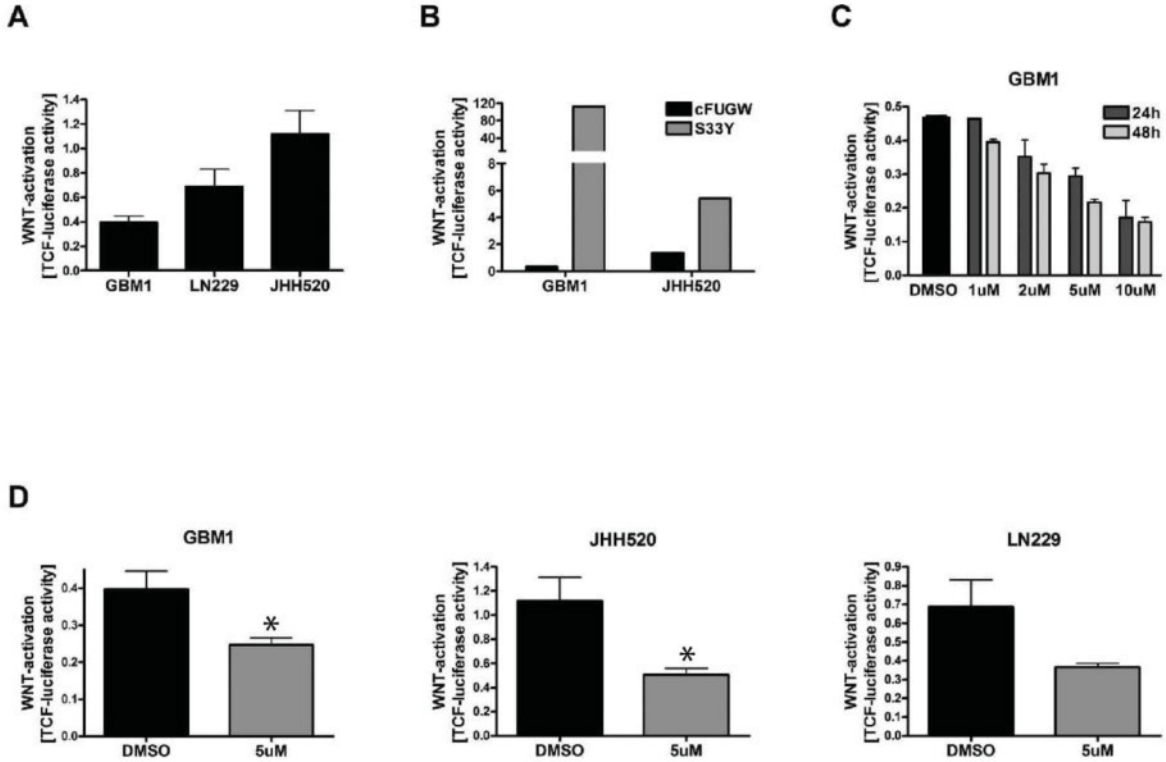


FIGURE 3. LGK974 suppresses canonical Wnt activity. **(A)** Wnt pathway activity assessed by TCF luciferase reporters. **(B)** Introduction of mutant active CTNNB1 (*S33Y*) increased TCF reporter signals in GBM1 and JHH520 cells. **(C)** LGK974 inhibits Wnt activity in a dose-dependent manner. **(D)** Treatment with 5 μmol/L LGK974 for 48 hours effectively inhibited Wnt signaling in all glioma cell lines tested (* $p \leq 0.05$). DMSO, dimethyl sulfoxide.

were correlated with available overall survival data provided by TCGA, with a cutoff of 1.75 SD above the median (*z*-score) considered as AXIN2 high-expressing tumors.

Fluorescence-Activated Cell Separation and Analyses

For CD133 cell surface immunostaining, 5×10^5 cells were retrieved from a single-cell suspension and labeled with anti-CD133/1-phycoerythrin antibody (AC133, no. 130-080-801; Miltenyi Biotec, Cologne, Germany) according to the manufacturer's instructions. The antibody solution consisted of anti-CD133/1-phycoerythrin (1:11) plus FcR blocking reagent (1:11, no. 130-059-901; Miltenyi Biotec) in 1× PBS (Life Technologies). All CD133-positive fractions were gated using the respective controls (AC133-pure, no. 130-090-422; Miltenyi Biotec). Fluorescence-activated cell analyses were performed on Accuri C6 (BD Biosciences, Franklin Lakes, NJ). Fluorescence-activated cell sorting was performed on SH800 (Sony Biosciences, Cambridge, MA), and FlowJo V10 software (Tree Star Inc, Ashland, OR) was applied to perform data postprocessing.

Cell Growth, Proliferation, and Apoptosis Assays

Cultures were dissociated into a single-cell suspension, and viable cells were quantified using the MUSE Count and Viability Assay Kit (no. MCH100102; Merck KGaA, Darmstadt, Germany); 2,000 cells/well (96-well plates) were plated in 100-μL triplicates. For this assay, GBM1 and JHH520 were grown as adherent cells on plates coated with laminin (no. L2020; Sigma-Aldrich; minimum of 3 hours prior cell plating with 20 μg/mL laminin solution) under stem cell culture conditions and replaced with fresh media with consistent drug concentration (5 μmol/L) every 48 hours. Relative cell numbers were measured at 1, 2, 3, and 4 days using the fluorescence viable cell mass assay TiterBlue, according to the manufacturer's instructions (no. G8081; Promega), on the Infinite M1000Pro plate reader (Tecan). Cell TiterBlue reagent was added directly to the cells (20 μL per well) and incubated for 2 hours at 37°C. Fluorescence intensity was measured at $560_{ex}/590_{em}$ nm.

To assess the effects of drug treatment on cell proliferation, we performed Ki67quantification on Days 2 and 3 after initiation of pharmacologic treatment using the Muse Ki67 Proliferation Kit (no. MCH100114; Merck KGaA) on the Muse Cell Analyzer (no. 0500-3115; Merck KGaA). Reduction of proliferation was normalized to dimethyl sulfoxide–treated control, and Ki67 stain was gated with the respective IgG stain control. For each sample, 50,000 cells were stained for 45 minutes.

Apoptotic cells were quantified using the Annexin V and Dead Cell Kit (no. MCH100105; Merck KGaA) on the Muse Cell Analyzer according to the manufacturer's protocol. A minimum of 2,000 gated events were acquired.

Colony Formation Assay in Soft Agarose

Six-well plates were coated with a bottom agar/media layer made from a 1:1 mixture of a prepared 2× concentration of neurosphere media and 1% melted agarose (Life Technologies) in water. A single-cell triturated suspension was placed into a top agarose/media mixture (0.7%) and immediately plated into 6-well plates at densities of 3,500 cells/well (GBM1) and 5,000 cells/well (JHH520) in 1.5 mL of agarose; 1.5 mL of media (supplemented with drug or vehicle) was placed into each well. For the effects of LGK974 on colony formation, 500 mL of fresh neurosphere media (with and without drug) was added to the treatment group every 48 hours. The experiment was stopped by viable cell visualization with nitroblue tetrazolium (Sigma-Aldrich) overnight at 37°C on Day 19 and quantified using MCID Elite software (MCID, Cambridge, United Kingdom).

Statistical Evaluation

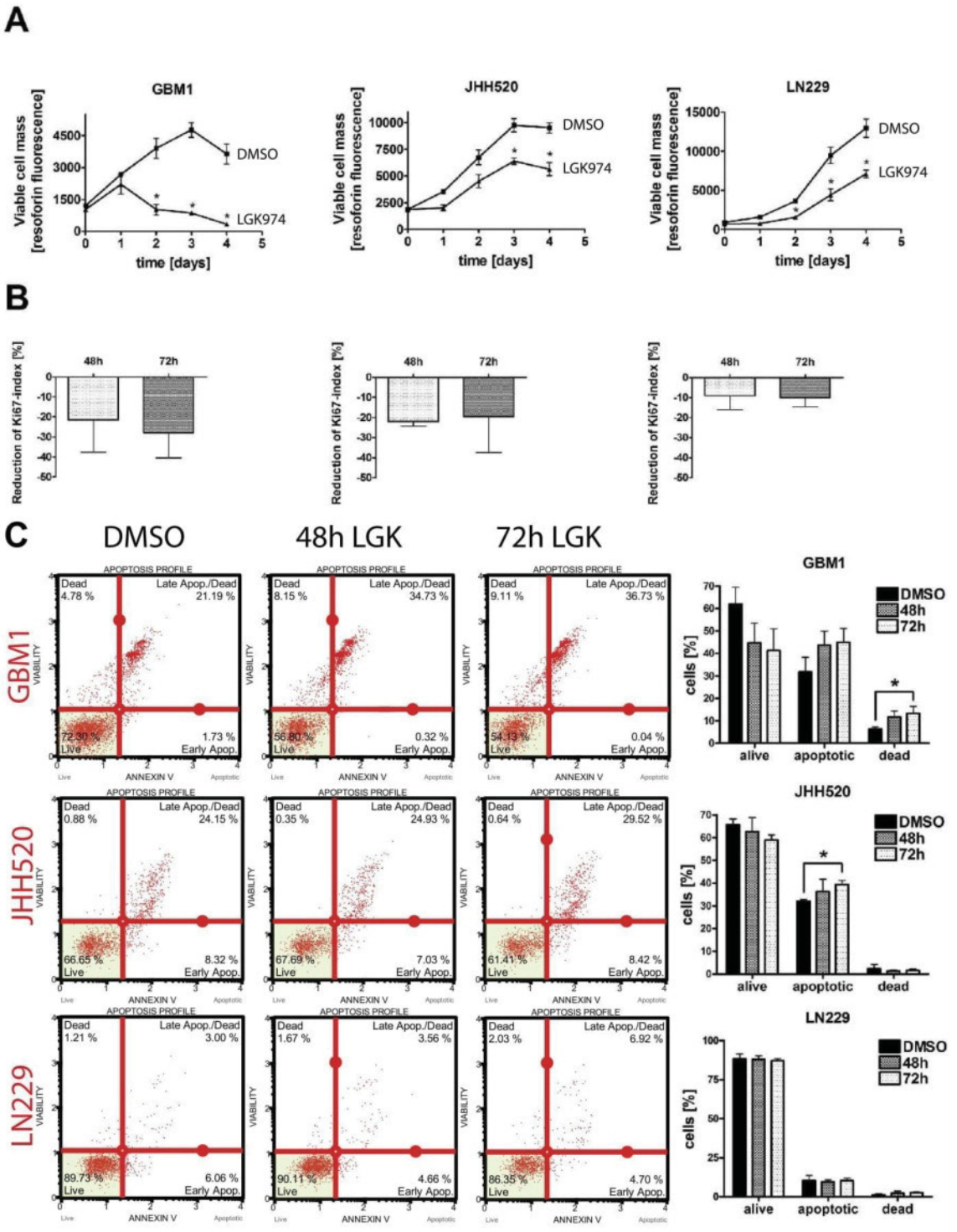
Kaplan-Meier analysis using logrank test compared overall survival between groups and was performed with Prism version 4 (GraphPad Software Inc, La Jolla, CA). Statistical analyses of in vitro experiments, which each included a minimum of 3 biologic replicates, were performed using 2-tailed Student *t*-test and Statistica software (Statsoft, Tulsa, OK). Data are presented as mean ± SD, and $p \leq 0.05$ was considered significant.

RESULTS

Levels of Wnt Activity in Surgical GBM Specimens and Glioma Cell Lines

We first used immunohistochemistry to examine the expression and localization of CTNNB1/β-catenin in surgical GBM specimens and in cell pellets from the GBM1 neurosphere line that were fixed and processed in parallel with the clinical samples. Among the primary tumors in our tissue microarray, 30 pediatric and 43 adult glioblastoma samples had sufficient material on stained slides for scoring. Among these, the degree of cytoplasmic and cell membrane CTNNB1 protein expression varied widely, with most cases showing no or weak expression; however, approximately one quarter showed moderate or strong expression (Figs. 1A, B). We did not identify the type of strong nuclear protein reported in tumors such as medulloblastoma, in which the pathway is activated by *CTNNB1* mutation (62, 63). However, in 8 of 43 adult GBM samples (19%) (Fig. 1A) and in 9 of 30 pediatric GBM samples (30%) (Fig. 1B), we detected weak immunoreactivity in a subset of nuclei, which could potentially represent pathway activity. The presence of weak nuclear staining was seen in cases with a range of cytoplasmic expression, and the 2 did not seem to correlate. GBM1 neurosphere cells showed weak cytoplasmic staining levels similar to many GBM but no evidence of nuclear CTNNB1 (Fig. 1C).

The relationship between protein expression and clinical outcome was also evaluated. Patients with GBM showing nuclear CTNNB1 in their tumors had a median survival of 17 months compared with 20 months for those without intranuclear staining. Logrank analysis of Kaplan-Meier survival curves revealed that this difference was not significant (Fig. 1D). Examination of the prognostic impact of nuclear CTNNB1 on adult and pediatric cases individually revealed equal survival in adults (20 vs 20 months) but shorter survival in patients younger than 18 years with nuclear protein (14 vs 20 months), although the difference was not significant even



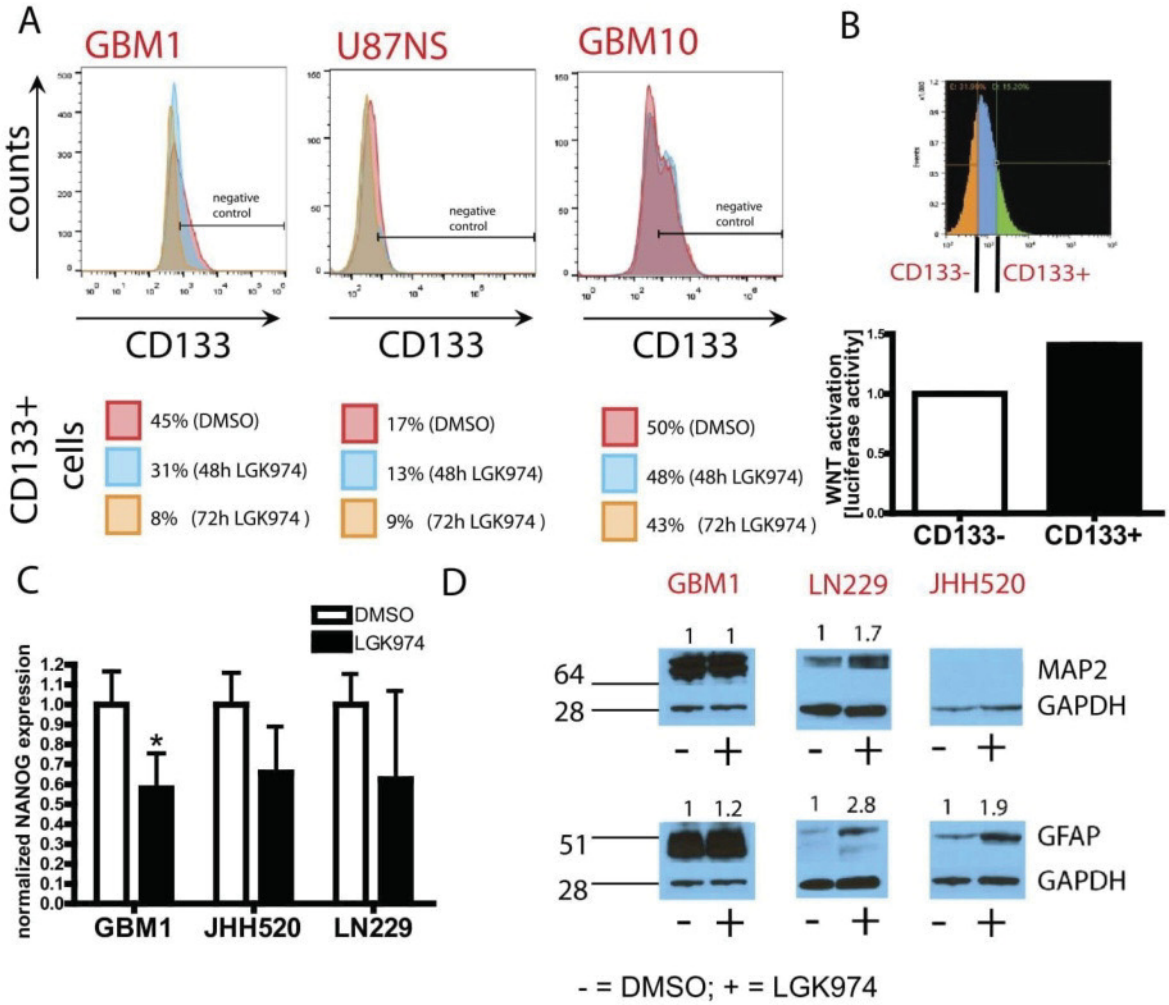


FIGURE 5. LGK974 promotes differentiation. **(A)** LGK974 treatment reduced the fraction of cells expressing the GSC marker CD133. **(B)** CD133-positive GBM1 cells have higher canonical Wnt activity compared with their CD133-negative counterparts. **(C)** Reduced expression of NANOG after LGK974 administration (* p = 0.05). **(D)** Increased glial fibrillary acidic protein (all tested cell lines) and microtubule-associated protein 2 (MAP2) (LN229) 72 hours after administration of LGK974. DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

in these pediatric patients. Analyses of adult GBM and pediatric GBM with and without cytoplasmic CTNNB1 protein did not reveal any survival differences between patient groups. We also did not identify any correlation between cytoplasmic or nuclear CTNNB1 and expression of mutant *IDH1*, as detected by immunohistochemistry. These findings suggest that oncogenic Wnt signaling is active in a subset of GBM, but a possible association with worse clinical outcomes is not clear. Because immunohistochemical analysis was difficult owing to weak nuclear CTNNB1 staining, we sought to use more quantitative and sensitive methods to assess Wnt signaling status.

Expression of AXIN2, an established target of canonical Wnt signaling (34, 40, 44, 49, 64), has been shown to be

FIGURE 4. Decreased growth after Wnt pathway blockade. Wnt inhibition significantly reduced cell growth, as assessed with TiterBlue assay **(A)**; cell proliferation, as assessed by fluorescence-based Ki67 quantification **(B)**; and cell survival, as assessed with Annexin V/propidium iodide–based apoptosis and cell death quantification **(C)**. * $p \leq 0.05$ for all panels. 48h, 48 hours; 72h, 72 hours; DMSO, dimethyl sulfoxide.

associated with Wnt activity and glioma stemness (42–44, 65). Therefore, we measured AXIN2 to determine whether the brain tumor cell lines used in our laboratory had levels of pathway activity similar to those found in snap-frozen patient specimens. As shown in Figure 2A, AXIN2 mRNA levels in the 6 adult tumor specimens examined (adult GBM: p349, p635, p636, p696, and p770; low-grade glioma: p824) varied more than 2-fold between tumors. AXIN2 levels were even more heterogeneous in in vitro models, including cell lines derived from 9 adult GBM tumors (GBM1, GBM10, GBM14, JHH136, JHH520, AQH612, U87, U87NS, and LN229) and 9 pediatric brain tumors, including 1 diffuse intrinsic pontine glioma (SU-DIPG) (66), 1 anaplastic astrocytoma (BT35) (67), 1 malignant atypical teratoid rhabdoid tumor (BT40) (67), 2 low-grade gliomas (Res186 and Res259) (68), 2 high-grade gliomas (KNS42 (59) and SF188 (69)), and 2 medulloblastomas (D283 and D425) (70).

We noted no clear correlation between AXIN2 expression levels in our cell lines and patient age or tumor type. However, a number of GBM lines showed levels of AXIN2 similar to those seen in primary tumors. We selected 2 neurosphere lines (GBM1 and JHH520) and 1 adherent line (LN229) that showed relatively high AXIN2 expression within the physiologically relevant range for further studies of functional inhibition.

We also examined AXIN2 mRNA levels as a potential prognostic marker in 396 GBM specimens from the database of TCGA, with follow-up data available. A number of potential thresholds for AXIN2 overexpression were tested; however, although shorter survival was associated with higher levels of this Wnt pathway target, the differences were not statistically significant. The most prominent effects were noted with the quite stringent thresholds for AXIN2 overexpression. Figure 2B shows that patients with high AXIN2 (defined as expression $\geq$1.75-fold SD above the median) survived 208 versus 448 days for the rest of the TCGA group ($p = 0.06$).

Wnt Pathway Luciferase Reporter Assays in Cell Line Models

Luciferase reporter systems driven by CTNNB1/β-catenin binding to multimerized TCF/LEF promoter sites are frequently used to measure canonical Wnt activity in cell lines. We introduced this reporter into our GBM lines using lentivirus and selected for stable integration using puromycin. Interestingly, TCF/LEF reporter signals correlated with AXIN2 mRNA levels, with moderate luciferase signals in GBM1 (0.4), with higher luciferase signals in LN229 (0.7), and with the highest luciferase signals in JHH520 (1.1) (Fig. 3A), supporting AXIN2 as a marker of Wnt activity in these tumors. When GBM1 and JHH520 reporter cultures were transduced with constitutively active (*S33Y* mutant) CTNNB1/β-catenin (34), we observed a more than 100-fold (GBM1) or 4-fold (JHH520) induction of luciferase activity (Fig. 3B), confirming the responsiveness of the reporter.

LGK974 Treatment Reduces Wnt Transcriptional Activity and Cell Growth in GBM Cells

We next tested the effects of LGK974 administration on Wnt transcriptional activity in our culture models. In GBM1 cells, we identified 5 μmol/L LGK974 as sufficient to suppress canonical Wnt signaling; a representative experiment is shown in Figure 3C. Additional experiments using 5 μmol/L LGK974 in GBM1 and other lines confirmed 40% to 60% Wnt pathway suppression, which was significant in 2 lines ($p \leq 0.05$) (Fig. 3D). We chose to use this dose for functional studies to avoid potential nonspecific cytotoxicity.

Glioma cells with suppressed Wnt signaling showed reduced overall growth compared with their vehicle (i.e. dimethyl sulfoxide)–treated counterparts, as assessed with Titer-Blue assay. Statistical significance was reached at 48 hours of treatment (GBM1 and LN229) and at 96 hours of treatment (JHH520) (Fig. 4A). We also tested U87 and SF188 (2 cell lines with low AXIN2 baseline levels, as shown in Fig. 2) but did not see significant growth inhibition after LGK974 treatment (data not shown), suggesting that Wnt activity might be predictive of susceptibility to pathway inhibition.

LGK974-sensitive lines showed a reduction in the percentage of proliferating cells, as assessed with fluorescence-based quantification of Ki67 expression (Fig. 4B). An increase in apoptotic cell death, as detected by fluorescence-based quantification of Annexin V/propidium iodide–positive cells, was also seen in GBM1 and JHH520 after 48 and 72 hours of LGK974 treatment; however, no induction of apoptosis was observed in LN229 (Fig. 4C).

LGK974 Treatment Reduces In Vitro Clonogenicity and Induces Glial and Neural Differentiation

Canonical Wnt signaling has been implicated as a regulator of the GSC marker CD133 and has been shown to affect glioma cell differentiation (13, 34). Glioblastoma multiformecultures expressing the cell surface marker CD133 showed a reduction in the percentage of positive cells after treatment with LGK974 (GBM1: 45%–8%; U87NS: 17%–9%; GBM10: 50%–43%) (Fig. 5A). The JHH520 and LN229 lines had very low baseline levels of CD133-positive cells (approximately 1%–2%), and this did not change significantly after LGK974 exposure (data not shown). When we sorted GBM1 cultures by CD133 expression using fluorescence-activated cell sorting, the positive stem-like fraction had somewhat higher levels of Wnt signaling, as assessed by the TCF luciferase reporter, although the pathway was clearly also active in better differentiated cells (Fig. 5B). LGK974 also reduced mRNA levels of the neural stem cell marker NANOG in all tested lines, reaching significance for GBM1 (Fig. 5C).

Because induction of differentiation can be an effective anticancer stem cell therapeutic strategy for malignant gliomas (71), we also assessed whether Wnt blockade could promote differentiation. Western blot analysis revealed induction of the glial differentiation marker glial fibrillary acidic protein in LN229 and JH520 and a very minor increase in GBM1 (Fig. 5C). The neuronal marker microtubule-associated protein 2 was only induced in LN229 after Wnt inhibition (Fig. 5D).

Given the reductions in the stem-like cell fraction, we evaluated the effects of LGK974 treatment on in vitro clonogenicity. Soft agar colony formation assays showed a

significant reduction in total number of spheres formed and mean sphere diameter after drug exposure in both GBM1 and JHH520 neurosphere lines ($p \leq 0.001$). However, the magnitude of the effect was much more prominent in GBM1, with a mean sphere number reduction of more than 80% and a mean colony size decrease from 147 to 86 μm (Fig. 6).

DISCUSSION

Recent studies have identified molecular changes affecting the Wnt pathway in malignant gliomas (33, 72–74). These and other research studies have suggested that ligand-driven upregulation of Wnt is involved in glioma pathogenesis. For example, Wnt3a can control tumorigenicity through regulation of the cell cycle regulator FoxM1, which promotes intranuclear accumulation of CTNNB1/β-catenin (44). In addition, ectopic expression of soluble Frizzled-related proteins—secreted factors that interact with and control Wnt ligands (75)—inhibits glioma cell motility (76). The Wnt-specific secretory protein EVI/Wntless, which controls the secretion of pathway ligands in canonical and noncanonical contexts, is overexpressed in high-grade brain tumors and promotes GBM malignancy (46). The expression of Wnt inhibitory factor 1, a soluble inhibitor of Wnt morphogens, is downregulated in GBM and thereby activates the Wnt network, promoting tumor invasion (36). Given these data indicating that Wnt signaling in gliomas can be activated at the ligand level, we investigated the effects of LGK974 (51), an inhibitor of palmitoylation and extracellular secretion of Wnt ligands (77), on adult glioma cells in vitro.

Using cell lines grown adherently or in serum-free media as neurospheres, we found that LGK974 could suppress canonical Wnt activity in a dose-dependent manner, as measured by a highly sensitive luciferase-based reporter of transcriptional activity for the pathway. Wnt pathway suppression was associated with significant reductions in the proliferation index and in the overall growth of all 3 lines tested. We also noted increased glial differentiation, as evidenced by a stronger expression of glial fibrillary acidic protein, and a reduction in NANOG levels and in the percentage of cells

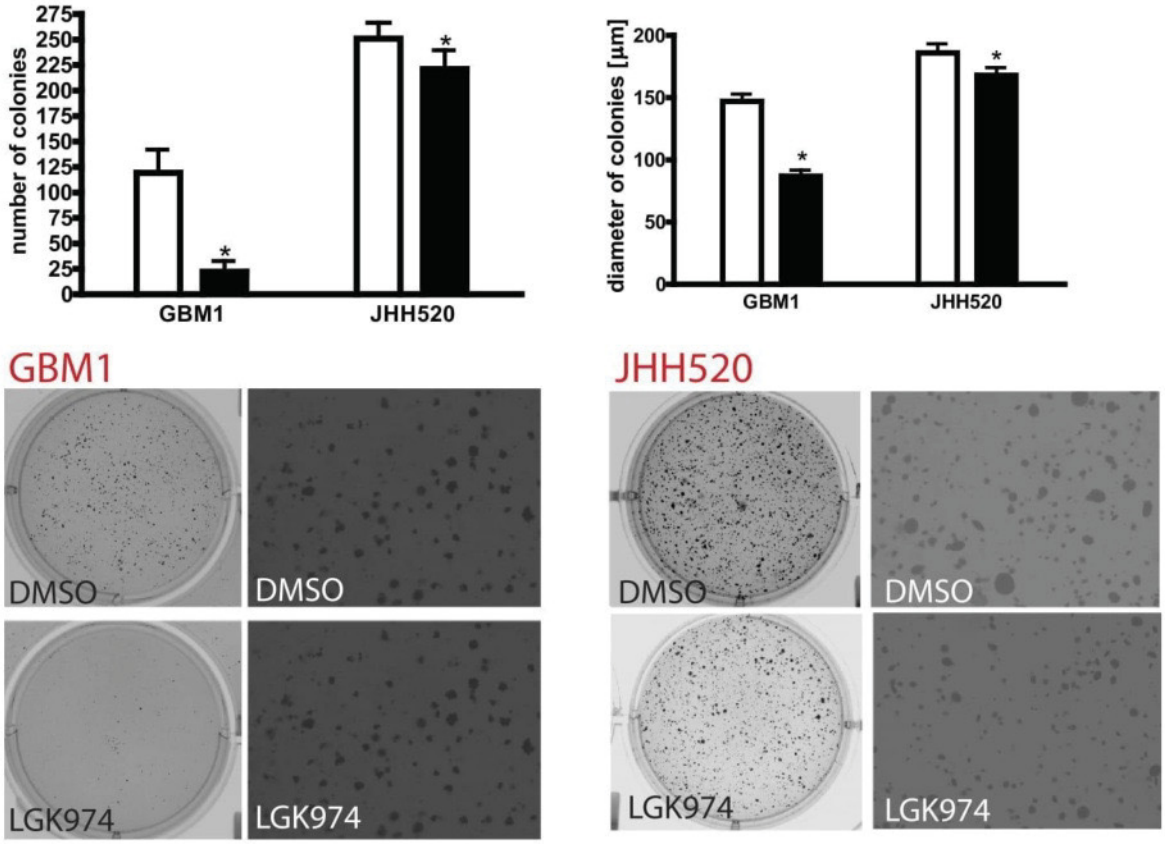


FIGURE 6. LGK974 treatment significantly inhibits in vitro clonogenicity and reduces mean sphere diameter (GBM1: from 147 to 87 μm; JHH520: from 186 to 168 μm) compared with dimethyl sulfoxide (DMSO)–treated control cells (* $p \leq 0.001$ for both parameters in both cell lines).

expressing the stem cell marker CD133. Consistent with the notion that a stem-like fraction was being depleted, Wnt inhibition caused a decrease in the size and number of colonies formed in soft agar.

To assess the extent of Wnt activity in primary gliomas, we examined the expression and localization of CTNNB1/β-catenin protein in 73 pediatric and adult surgical GBM specimens because nuclear translocation of this protein is associated with canonical signaling (62). Nuclear CTNNB1 was identified in 30% of pediatric GBM samples and 19% of adult GBM samples, but expression was weak and present in relatively small numbers of cells. This assay did not show nuclear CTNNB1 in GBM1 cultures, suggesting that the immunohistochemical assay is less sensitive than TCF luciferase reporters, which identified biologically significant Wnt activity in these cells. The weak nuclear staining may also reflect a more modest ligand-driven activation of Wnt signaling in gliomas, as opposed to *adenomatous polyposis coli* loss or *CTNNB1* mutations, which activate the pathway in medulloblastoma (78). Nevertheless, together with prior reports, our findings support the concept that Wnt is active in a significant number of GBM and may be particularly frequent in pediatric tumors.

Several earlier studies found that increased cytoplasmic CTNNB1/β-catenin protein levels and nuclear translocation could be prognostically significant in patients with gliomas. Liu et al (79) reported that 28% of the 43 glioblastomas they examined had cytoplasmic and nuclear proteins, which were associated with significantly shorter survival. Other groups showed that increased cytoplasmic or nuclear CTNNB1/β-catenin was associated with higher glioma grade and poor outcomes (80, 81). In contrast, Zhang et al (82) found in a series of 63 astrocytomas that only 4 cases showed nuclear β-catenin immunoreactivity and that, although increased overall protein levels were not associated with tumor grade, they did correlate with shorter survival. Finally, the presence of nuclear Y333 phosphorylated β-catenin in GBM cells has been linked to shorter survival in a series of 84 patients (14).

Although we found a shortened overall survival in patients with GBM whose tumors had nuclear CTNNB1 (17 vs 20 months), this difference was not significant. When we analyzed only patients younger than 18 years, the survival disadvantage of nuclear CTNNB1 was more pronounced (14 vs 20 months) but still not significant. Potential causes of the differences between our clinical correlation data and those from other groups include variations in staining protocols, scoring of combined nuclear and cytoplasmic proteins in some studies, and the small size and mixed nature of many previously published clinical cohorts. We also examined the prognostic impact of the Wnt target gene *AXIN2*. As was seen for nuclear CTNNB1, increased Wnt activity, as defined by high AXIN2 levels, was associated with shorter survival, but this was not significant.

Despite the proven oncogenic role of Wnt in gliomas (14, 15, 44, 83) and some progress in the development of pathway inhibitors (84, 85), little has been published on small-molecule compounds targeting Wnt, which are ready for clinical use in gliomas. One recent study tested the AXIN stabilizer SEN461, which showed promising therapeutic effects and inhibited Wnt activity in GBM cells in vitro and in vivo (86). Lan et al (87) used aspirin to suppress both Wnt signaling and the invasion and survival of GBM cells, although this anti-inflammatory agent can have a variety of effects. Another group investigated the antipsychotic drug risperidone, which decreased CTNNB1 in gliomas and reduced the stem-like cell fraction (88). FH535, a small-molecule Wnt inhibitor, suppressed Wnt activity in U87 and LN229 cells and reduced cellular invasion and proliferation (81). Finally, the use of genetic constructs to modulate the epigenome regulating Wnt—that is, miR-34a (89), miR-92b (90), miR-96 (74), miR-218 (35), miR-328 (91), and miR-603 (33)—is also possible, but efficient in vivo delivery remains challenging.

In summary, our data support the concept that Wnt signaling is active in at least a subset of malignant gliomas and that inhibition of this pathway can slow tumor growth, reduce the stem-like cellular fraction, and block clonogenicity in some GBM neurosphere lines. Our findings also suggest that the porcupine inhibitor LGK974, which is currently in trial for pancreatic and colorectal cancers, can effectively suppress Wnt signaling in GBM in vitro. However, preclinical in vivo studies must be performed to assess the therapeutic potential of LGK974 in the treatment of brain tumors.

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REFERENCES

1. Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005;352:987–96
2. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. Nature 2004;432:396–401
3. Binda E, Reynolds BA, Vescovi AL. Glioma stem cells: Turpis omen in nomen? (The evil in the name?). J Intern Med 2014;276:25–40
4. Galli R, Binda E, Orfanelli U, et al. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. Cancer Res 2004;64:7011–21
5. Zheng H, Ying H, Yan H, et al. p53 and Pten control neural and glioma stem/progenitor cell renewal and differentiation. Nature 2008;455:1129–33
6. Lagadec C, Vlashi E, Frohnen P, Alhiyari Y, Chan M, Pajonk F. The RNA-binding protein Musashi-1 regulates proteasome subunit expression in breast cancer– and glioma-initiating cells. Stem Cells 2014;32:135–44
7. Jin X, Jin X, Jung JE, Beck S, Kim H. Cell surface nestin is a biomarker for glioma stem cells. Biochem Biophys Res Commun 2013;433:496–501
8. Zbinden M, Duquet A, Lorente-Trigos A, Ngwabyt SN, Borges I, Ruiz i Altaba A. NANOG regulates glioma stem cells and is essential in vivo acting in a cross-functional network with GLI1 and p53. EMBO J 2010;29:2659–74
9. Gangemi RMR, Griffero F, Marubbi D, et al. SOX2 silencing in glioblastoma tumor–initiating cells causes stop of proliferation and loss of tumorigenicity. Stem Cells 2009;27:40–48
10. Bao S, Wu Q, McLendon RE, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 2006;444:756–60

11. Schonberg DL, Lubelski D, Miller TE, Rich JN. Brain tumor stem cells: Molecular characteristics and their impact on therapy. Mol Aspects Med 2014;39:82–101
12. Auffinger B, Spencer D, Pytel P, Ahmed AU, Lesniak MS. The role of glioma stem cells in chemotherapy resistance and glioblastoma multiforme recurrence. Expert Rev Neurother 2015:1–12
13. Rampazzo E, Persano L, Pistollato F, et al. Wnt activation promotes neuronal differentiation of glioblastoma. Cell Death Dis 2013;4:e500
14. Yang W, Xia Y, Ji H, et al. Nuclear PKM2 regulates β-catenin transactivation upon EGFR activation. Nature 2011;480:118–22
15. Zheng H, Ying H, Wiedemeyer R, et al. PLAGL2 regulates Wnt signaling to impede differentiation in neural stem cells and gliomas. Cancer Cell 2010;17:497–509
16. Gong A, Huang S. FoxM1 and Wnt/β-catenin signaling in glioma stem cells. Cancer Res 2012;72:5658–62
17. Kahlert UD, Nikkhah G, Maciaczyk J. Epithelial-to-mesenchymal(–like) transition as a relevant molecular event in malignant gliomas. Cancer Lett 2013;331:131–38
18. MacDonald BT, Tamai K, He X. Wnt/β-catenin signaling: Components, mechanisms, and diseases. Dev Cell 2009;17:9–26
19. Clevers H. Wnt/beta-catenin signaling in development and disease. Cell 2006;127:469–80
20. Ring A, Kim Y-M, Kahn M. Wnt/catenin signaling in adult stem cell physiology and disease. Stem Cell Rev 2014;10:512–25
21. Sonderegger S, Pollheimer J, Knöfler M. Wnt signalling in implantation, decidualisation and placental differentiation—review. Placenta 2010;31:839–47
22. Miki T, Yasuda S, Kahn M. Wnt/β-catenin signaling in embryonic stem cell self-renewal and somatic cell reprogramming. Stem Cell Rev 2011;7:836–46
23. Nusse R. Wnt signaling and stem cell control. Cell Res 2008;18:523–27
24. Pinto D, Gregorieff A, Begthel H, Clevers H. Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. Genes Dev 2003;17:1709–13
25. Kalani MYS, Cheshier SH, Cord BJ, et al. Wnt-mediated self-renewal of neural stem/progenitor cells. Proc Natl Acad Sci U S A 2008;105:16970–75
26. Zechner D, Fujita Y, Hülsken J, et al. Beta-catenin signals regulate cell growth and the balance between progenitor cell expansion and differentiation in the nervous system. Dev Biol 2003;258:406–18
27. Wexler EM, Paucer A, Kornblum HI, Palmer TD, Geschwind DH. Endogenous Wnt signaling maintains neural progenitor cell potency. Stem Cells 2009;27:1130–41
28. Mikels AJ, Nusse R. Wnts as ligands: Processing, secretion and reception. Oncogene 2006;25:7461–68
29. Buechling T, Boutros M. Wnt signaling at and above the receptor level. Curr Top Dev Biol 2011;97:21–53
30. Gao C, Chen YG. Dishevelled: The hub of Wnt signaling. Cell Signal 2010;22:717–27
31. Pu P, Zhang Z, Kang C, et al. Downregulation of Wnt2 and beta-catenin by siRNA suppresses malignant glioma cell growth. Cancer Gene Ther 2009;16:351–61
32. Jiang X, Yu Y, Yang HW, Agar NY, Frado L, Johnson MD. The imprinted gene *PEG3* inhibits Wnt signaling and regulates glioma growth. J Biol Chem 2010;285:8472–80
33. Guo M, Zhang X, Wang G, et al. miR-603 promotes glioma cell growth via Wnt/β-catenin pathway by inhibiting WIF1 and CTNNBIP1. Cancer Lett 2015;360:76–86
34. Kahlert UD, Maciaczyk D, Doostkam S, et al. Activation of canonical WNT/β-catenin signaling enhances in vitro motility of glioblastoma cells by activation of ZEB1 and other activators of epithelial-to-mesenchymal transition. Cancer Lett 2012;325:42–53
35. Tu Y, Gao X, Li G, et al. MicroRNA-218 inhibits glioma invasion, migration, proliferation, and cancer stem-like cell self-renewal by targeting the polycomb group gene *Bmi1*. Cancer Res 2013;73:6046–55
36. Vassallo I, Zinn P, Lai M, Rajakannu P, Hamou MF, Hegi ME. WIF1 re-expression in glioblastoma inhibits migration through attenuation of non-canonical WNT signaling by downregulating the lncRNA MALAT1. Oncogene 2015. doi: 10.1038/onc.2015.61 [Epub ahead of print]
37. Kim Y, Kim KH, Lee J, et al. Wnt activation is implicated in glioblastoma radioresistance. Lab Invest 2012;92:466–73
38. Kaur N, Chettiar S, Rathod S, et al. Wnt3a mediated activation of Wnt/β-catenin signaling promotes tumor progression in glioblastoma. Mol Cell Neurosci 2013;54:44–57
39. Jho E, Zhang T, Domon C, Joo CK, Freund JN, Costantini F. Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol Cell Biol 2002;22:1172–83
40. Leung JY, Kolligs FT, Wu R, et al. Activation of AXIN2 expression by beta-catenin–T cell factor. A feedback repressor pathway regulating Wnt signaling. J Biol Chem 2002;277:21657–65
41. Chia IV, Costantini F. Mouse axin and axin2/conductin proteins are functionally equivalent in vivo. Mol Cell Biol 2005;25:4371–76
42. Lambiv WL, Vassallo I, Delorenzi M, et al. The Wnt inhibitory factor 1 (WIF1) is targeted in glioblastoma and has a tumor suppressing function potentially by induction of senescence. Neuro-oncology 2011;13:736–47
43. Zhang W, Shen C, Li C, et al. miR-577 inhibits glioblastoma tumor growth via the Wnt signaling pathway. Mol Carcinog 2015. doi: 10.1002/mc.22304 [Epub ahead of print]
44. Zhang N, Wei P, Gong A, et al. FoxM1 promotes β-catenin nuclear localization and controls Wnt target-gene expression and glioma tumorigenesis. Cancer Cell 2011;20:427–42
45. Götze S, Wolter M, Reifenberger G, Müller O, Sievers S. Frequent promoter hypermethylation of Wnt pathway inhibitor genes in malignant astrocytic gliomas. Int J Cancer 2010;126:2584–93
46. Augustin I, Goidts V, Bongers A, et al. The Wnt secretion protein Evi/Gpr177 promotes glioma tumourigenesis. EMBO Mol Med 2012;4:38–51
47. Kamino M, Kishida M, Kibe T, et al. Wnt-5a signaling is correlated with infiltrative activity in human glioma by inducing cellular migration and MMP-2. Cancer Sci 2011;102:540–48
48. Yu JM, Jun ES, Jung JS, et al. Role of Wnt5a in the proliferation of human glioblastoma cells. Cancer Lett 2007;257:172–81
49. Jin X, Jeon H-Y, Joo KM, et al. Frizzled 4 regulates stemness and invasiveness of migrating glioma cells established by serial intracranial transplantation. Cancer Res 2011;71:3066–75
50. Zheng Y, McFarland BC, Drygin D, et al. Targeting protein kinase CK2 suppresses prosurvival signaling pathways and growth of glioblastoma. Clin Cancer Res 2013;19:6484–94
51. Liu J, Pan S, Hsieh MH, et al. Targeting Wnt-driven cancer through the inhibition of Porcupine by LGK974. Proc Natl Acad Sci U S A 2013;110:20224–29
52. Jiang X, Hao H-X, Growney JD, et al. Inactivating mutations of RNF43 confer Wnt dependency in pancreatic ductal adenocarcinoma. Proc Natl Acad Sci U S A 2013;110:12649–54
53. Tzeng H-E, Yang L, Chen K, et al. The pan-PI3K inhibitor GDC-0941 activates canonical WNT signaling to confer resistance in TNBC cells: Resistance reversal with WNT inhibitor. Oncotarget 2015;6:11061–73
54. Kahlert UD, Bender NO, Maciaczyk D, et al. CD133/CD15 defines distinct cell subpopulations with differential in vitro clonogenic activity and stem cell–related gene expression profile in in vitro propagated glioblastoma multiforme–derived cell line with a PNET-like component. Folia Neuropathol 2012;50:357–68
55. Heaphy CM, Schreck KC, Raabe E, et al. A glioblastoma neurosphere line with alternative lengthening of telomeres. Acta Neuropathol 2013;126:607–8
56. Ahsan S, Raabe EH, Haffner MC, et al. Increased 5-hydroxymethylcytosine and decreased 5-methylcytosine are indicators of global epigenetic dysregulation in diffuse intrinsic pontine glioma. Acta Neuropathol Commun 2014;2:59
57. Fuerer C, Nusse R. Lentiviral vectors to probe and manipulate the Wnt signaling pathway. PLoS One 2010;5:e9370
58. Dull T, Zufferey R, Kelly M, et al. A third-generation lentivirus vector with a conditional packaging system. J Virol 1998;72:8463–71
59. Kahlert UD, Suwala AK, Raabe EH, et al. ZEB1 promotes invasion in human fetal neural stem cells and hypoxic glioma neurospheres. Brain Pathol 2014. doi: 10.1111/bpa.12240 [Epub ahead of print]
60. Mao X, Hütt-Cabezas M, Orr BA, et al. LIN28A facilitates the transformation of human neural stem cells and promotes glioblastoma tumorigenesis through a pro-invasive genetic program. Oncotarget 2013;4:1050–64

61. Gao J, Aksoy BA, Dogrusoz U, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 2013;6:pl1
62. Eberhart CG, Tihan T, Burger PC. Nuclear localization and mutation of beta-catenin in medulloblastomas. J Neuropathol Exp Neurol 2000;59: 333–37
63. Fattet S, Haberler C, Legoix P, et al. Beta-catenin status in paediatric medulloblastomas: Correlation of immunohistochemical expression with mutational status, genetic profiles, and clinical characteristics. J Pathol 2009;218:86–94
64. Schreck KC, Taylor P, Marchionni L, et al. The Notch target Hes1 directly modulates Gli1 expression and Hedgehog signaling: A potential mechanism of therapeutic resistance. Clin Cancer Res 2010;16:6060–70
65. Sareddy GR, Kesanakurti D, Kirti PB, Babu PP. Nonsteroidal anti-inflammatory drugs diclofenac and celecoxib attenuates Wnt/β-catenin/Tcf signaling pathway in human glioblastoma cells. Neurochem Res 2013;38:2313–22
66. Taylor KR, Mackay A, Truffaux N, et al. Recurrent activating ACVR1 mutations in diffuse intrinsic pontine glioma. Nat Genet 2014;46:457–61
67. Graham C, Tucker C, Creech J, et al. Evaluation of the antitumor efficacy, pharmacokinetics, and pharmacodynamics of the histone deacetylase inhibitor depsipeptide in childhood cancer models in vivo. Clin Cancer Res 2006;12:223–34
68. Brandt WD, Schreck KC, Bar EE, et al. Notch signaling activation in pediatric low-grade astrocytoma. J Neuropathol Exp Neurol 2015;74: 121–31
69. Trent J, Meltzer P, Rosenblum M, et al. Evidence for rearrangement, amplification, and expression of c-myc in a human glioblastoma. Proc Natl Acad Sci U S A 1986;83:470–73
70. Bai R, Siu I-M, Tyler BM, Staedtke V, Gallia GL, Riggins GJ. Evaluation of retinoic acid therapy for OTX2-positive medulloblastomas. Neuro-oncology 2010;12:655–63
71. Piccirillo SGM, Reynolds BA, Zanetti N, et al. Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour–initiating cells. Nature 2006;444:761–65
72. Liu H, Liu Z, Jiang B, Peng R, Ma Z, Lu J. SOX9 overexpression promotes glioma metastasis via Wnt/β-catenin signaling. Cell Biochem Biophys 2015. doi: 10.1007/s12013-015-0647-z [Epub ahead or print]
73. Zhang K, Zhu S, Liu Y, et al. ICAT inhibits glioblastoma cell proliferation by suppressing Wnt/β-catenin activity. Cancer Lett 2015;357:404–11
74. Yan Z, Wang J, Wang C, Jiao Y, Qi W, Che S. miR-96/HBP1/Wnt/β-catenin regulatory circuitry promotes glioma growth. FEBS Lett 2014;588:3038–46
75. Rattner A, Hsieh JC, Smallwood PM, et al. A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors. Proc Natl Acad Sci U S A 1997;94:2859–63
76. Roth W, Wild-Bode C, Platten M, et al. Secreted Frizzled-related proteins inhibit motility and promote growth of human malignant glioma cells. Oncogene 2000;19:4210–20
77. Willert K, Brown JD, Danenberg E, et al. Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature 2003;423:448–52
78. Paraf F, Jothy S, Van Meir EG. Brain tumor–polyposis syndrome: Two genetic diseases? J Clin Oncol 1997;15:2744–58
79. Liu C, Tu Y, Sun X, et al. Wnt/beta-catenin pathway in human glioma: Expression pattern and clinical/prognostic correlations. Clin Exp Med 2010;11:105–12
80. Yang C, Iyer RR, Yu ACH, et al. β-Catenin signaling initiates the activation of astrocytes and its dysregulation contributes to the pathogenesis of astrocytomas. Proc Natl Acad Sci U S A 2012;109:6963–68
81. Shi Z, Qian X, Li L, et al. Nuclear translocation of β-catenin is essential for glioma cell survival. J Neuroimmune Pharmacol 2012;7:892–903
82. Zhang L-Y, Jiang L-N, Li F-F, et al. Reduced beta-catenin expression is associated with good prognosis in astrocytoma. Pathol Oncol Res 2010; 16:253–57
83. Zhang K, Zhang J, Han L, Pu P, Kang C. Wnt/beta-catenin signaling in glioma. J Neuroimmune Pharmacol 2012;7:740–49
84. Voronkov A, Krauss S. Wnt/beta-catenin signaling and small molecule inhibitors. Curr Pharm Des 2013;19:634–64
85. Curtin JC, Lorenzi MV. Drug discovery approaches to target Wnt signaling in cancer stem cells. Oncotarget 2010;1:563–77
86. De Robertis A, Valensin S, Rossi M, et al. Identification and characterization of a small-molecule inhibitor of Wnt signaling in glioblastoma cells. Mol Cancer Ther 2013;12:1180–89
87. Lan F, Yue X, Han L, et al. Antitumor effect of aspirin in glioblastoma cells by modulation of β-catenin/T-cell factor–mediated transcriptional activity. J Neurosurg 2011;115:780–88
88. Peng H, Jiang B, Zhao J, Chen B, Wang P. Risperidone promotes differentiation of glioma stem-like cells through the Wnt signaling pathway. Tumour Biol 2015. doi: 10.1007/s13277-015-3087-x [Epub ahead of print]
89. Rathod SS, Rani SB, Khan M, Muzumdar D, Shiras A. Tumor suppressive miRNA-34a suppresses cell proliferation and tumor growth of glioma stem cells by targeting Akt and Wnt signaling pathways. FEBS Open Bio 2014;4:485–95
90. Li Q, Shen K, Zhao Y, Ma C, Liu J, Ma J. MiR-92b inhibitor promoted glioma cell apoptosis via targeting DKK3 and blocking the Wnt/β-catenin signaling pathway. J Transl Med 2013;11:302
91. Delic S, Lottmann N, Stelzl A, et al. MiR-328 promotes glioma cell invasion via SFRP1-dependent Wnt-signaling activation. Neuro-oncology 2014;16:179–90

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Research Paper

Inhibition of Wnt/beta-catenin signaling downregulates expression of aldehyde dehydrogenase isoform 3A1 (ALDH3A1) to reduce resistance against temozolomide in glioblastoma *in vitro*

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ABSTRACT

Glioblastoma is the most aggressive type of glioma. The Wingless (Wnt) signaling pathway has been shown to promote stem cell properties and resistance to radio- and chemotherapy in glioblastoma. Here, we demonstrate that pharmacological Wnt pathway inhibition using the porcupine inhibitor LGK974 acts synergistically with temozolomide (TMZ), the chemotherapeutic drug currently used as standard treatment for glioblastoma, to suppress *in vitro* growth of glioma cells. Synergistic growth inhibition was independent of the *O*^{6}-alkylguanine DNA alkyltransferase (*MGMT*) promoter methylation status. Transcriptomic analysis revealed that expression of aldehyde dehydrogenase 3A1 (*ALDH3A1*) was significantly down-regulated when cells were treated with LGK974 and TMZ. Suppressing ALDH3A1 expression increased the efficacy of TMZ and reduced clonogenic potential accompanied by decreased expression of stem cell markers CD133, Nestin and Sox2. Taken together, our study suggests that previous observations concerning Wnt signaling blockade to reduce chemoresistance in glioblastoma is at least in part mediated by inhibition of ALDH3A1.

INTRODUCTION

Glioblastoma is the most common primary malignant brain tumor in adults and is characterized by a dismal prognosis. Despite radical treatment with radio- and chemotherapy, the median overall survival is less than two years [1, 2]. One of the obstacles of curative treatment of glioblastoma is primary or acquired resistance to the current standard of care consisting of radiotherapy and chemotherapy with temozolomide (TMZ). In case of TMZ, which works as a DNA alkylating agent by adding alkyl-residues to the N-7 and O-6 positions of guanine, promoter methylation of the O-6-methylguanine-DNA methyltransferase (*MGMT*) gene is associated with more efficient therapeutic success [3]. TMZ is less effective in glioblastomas lacking *MGMT* promoter methylation, resulting in worse outcome of this group of patients [4]. New therapeutic options, such as anti-angiogenic strategies

employing blocking antibodies against vascular endothelial growth factor, did not result in significant overall survival benefit [5]. Therefore, other treatment targets must be identified and validated, with glioma stem-like cells (GSCs) having emerged as a promising target for future treatment. GSCs have been reported to be the most therapy resistant type of tumor cells in malignant gliomas, withstanding treatments with radio- and chemotherapy [6, 7]. Similar to somatic stem cells, GSCs are governed by deregulated phylogenetically conserved stem cell signaling pathways modulating differentiation, proliferation, invasion and stress regeneration capability [8]. One of these cascades is the Wingless (Wnt) pathway. Several drugs targeting various members of the Wnt signaling network have been developed and have shown promising preclinical results against various types of cancer including glioblastoma [9–13]. We have previously shown that pharmacological interference with the Wnt ligand-receptor interaction through inhibition of porcupine with LGK974 (Wnt974) is a novel strategy to efficiently block Wnt signaling activity in glioblastoma cells [14]. Here, we now demonstrate that LGK974 acts synergistically with TMZ chemotherapy to reduce cell viability in both *MGMT* promoter-methylated and unmethylated glioblastoma neurosphere cell lines. We found aldehyde dehydrogenase 3A1 (ALDH3A1), an enzyme involved in cellular metabolic clearance and detoxification of alcohol-derived acetaldehyde [15], to be down-regulated in cells treated with LGK974 and TMZ as compared to monotherapy with either TMZ or LGK974. Our results suggest that ALDH3A1 in glioblastoma is a target gene of the canonical Wnt signaling and we provide functional evidence that pharmacological inhibition of the pathway by porcupine inhibition increases susceptibly to TMZ treatment at least in part due to down-regulation of ALDH3A1. Therefore, targeting ALDH3A1 can be an innovative strategy to increase TMZ sensitivity in brain cancer cells independently of the *MGMT* promoter methylation status.

RESULTS

Pharmacological Wnt inhibition acts synergistically with TMZ to inhibit glioma cell growth

We characterized the *MGMT* promoter methylation status of four *in vitro* glioma models using methylation-specific PCR as either *MGMT* promoter-methylated (GBM1, JHH520) or -unmethylated (GBM10, SF188) supported by relatively low (GBM1, JHH520) or high (GBM10, SF188) IC_{50} values of TMZ (Table 1). IC_{50} concentrations for LGK974 and doses for γ-radiation were also determined for each cell line. In contrast to TMZ treatment, the *MGMT* promoter methylation status had no effect on therapy sensitivity when LGK974 or γ-radiation was applied to the cultures (Table 1). We previously showed that LGK974 effectively blocks canonical Wnt signaling activity in glioblastoma cells [14]. To assess whether LGK974 might increase sensitivity towards TMZ, we measured combinatory effects of both drugs by defining cell viability (using CellTiter Blue) as our primary readout and calculating synergistic effects due to combination index equation for multiple drug effect interactions using computerized simulations (Compusyn) [16]. We found that the combination of TMZ with LGK974 reduced cell growth significantly more effectively as compared to treatments with either drug alone irrespective on the *MGMT* promoter-methylation status (Figure 1A). The same effect was observed in cells treated with γ-radiation and LGK974 (Supplementary Figure 1A). Looking at the dose-effect curves for both drugs, we observed a sigmoidal curve for TMZ and a hyperbolic, non-linear relationship for LGK974 that explains diminishing increment of effectiveness as the concentration rises above the IC_{50} [17] (Supplementary Figure 1B). Stronger synergy was noticed in lower dosages of both drugs.

Base line ALDH3A1 expression is independent on Wnt pathway activity and TMZ resistance but is down-regulated upon treatment with LGK974 and TMZ

Next we performed the whole transcriptome analysis of GBM1 cells treated with DMSO control, TMZ, LGK974, and TMZ plus LGK974. We chose GBM1 for our analysis as we previously showed that LGK974 effectively reduces stemness in this cell line [14]. Evaluation of the data revealed significant down- or upregulation of 2175 genes with moderate fold change values in the combinatory treatment group as compared to the single treatment groups and the DMSO control group (Figure 1B). We validated the data by targeted expression analyses using quantitative real time PCR (qPCR) for nine differentially expressed genes. The nine genes were selected based on their relatively high overall expression values and the fold-change values of differential expression following combinatory treatment (Figure 1C, Supplementary Figure 2A). All genes showed similar expression tendencies as detected in the microarray screen, except HIST1H2BD, that showed an upregulation on the mRNA expression level (Supplementary Figure 2A). Thereby we confirmed that *ALDH3A1*, the strongest inhibited gene in the microarray screen, was robustly down-regulated by LGK974 and TMZ in GBM1 cells evaluated by real time-PCR. Moreover, we tested basal mRNA and protein expression of *ALDH3A1* of our cell lines and compared it with the specific IC_{50} dosage of TMZ (Figure 2A, Supplementary Figure 2B). We did not find any correlation between ALDH3A1 expression and resistance against TMZ, which is more predicted by

Table 1: IC_{50} doses for TMZ, LGK974 and γ-irradiation

Cell line	MGMT promoter	LGK974 [μM]	TMZ [μM]	γ-irradiation [Gy]
GBM1	methylated	1	5	2
JHH520	methylated	3	10	6
GBM10	unmethylated	6	70	2
SF188	unmethylated	1	40	2

Each cell line was treated with different dosages of TMZ and LGK974. Cell viability was taken as readout.

MGMT promoter methylation status. There was also no correlation found between ALDH3A1 expression and basal WNT activity in our cell lines (Supplementary Figure 2C).

Testing of *ALDH3A1* mRNA expression after pharmacological treatment in additional cell models SF188, JHH520 and GBM10 revealed that the latter two showed significant signal suppression. No difference in gene transcription was noticed in pediatric glioma model SF188 (Figure 2B). For these studies we used half dosages of the IC_{50} for each drug since these concentrations resulted in most significant synergistic effects (Figure 1A). The array data suggested that LGK974 treatment alone may not only down-regulate specific Wnt target genes such as Dickkopf3 and CD44, but also down-regulates expression of *ALDH3A1,* although to lesser extent as compared to combination treatment with TMZ (data not shown). Verifying this data using sensitive reporter readouts, we found combined TMZ and LGK974 treatment significantly reducing Wnt pathway activity in all four cell lines (Figure 2C). On protein level, combination treatment of LGK974 and TMZ reduced ALDH3A1 in GBM1 and SF188, whereas in GBM10 protein was reduced under LGK974 monotherapy only. No significant suppression of ALDH3A1 was seen in JHH520 (Figure 2D). In concordance, genetic inhibition of Wnt signaling in GBM1 cells using shRNA-mediated knock-down of β-catenin expression caused reduction in ALDH3A1 expression levels (Supplementary Figure 2D).

ALDH3A1 inhibition reduces cell viability and resistance to TMZ

To test whether ALDH3A1 mediates the resistance to TMZ, we created glioma cells with genetically down-regulated ALDH3A1 expression (Figure 3A). Most efficient KD was achieved in cell lines GBM1, GBM10 and SF188 and therefore chosen for further experiments. In comparison to control cells we noticed that cells with down-regulated ALDH3A1 expression grew slower and were significantly more sensitive to TMZ (Figure 3B, 3C). In contrast, ALDH3A1 knock-down cells did not alter their sensitivity towards LGK974 and combination treatment with TMZ (Figure 4A).

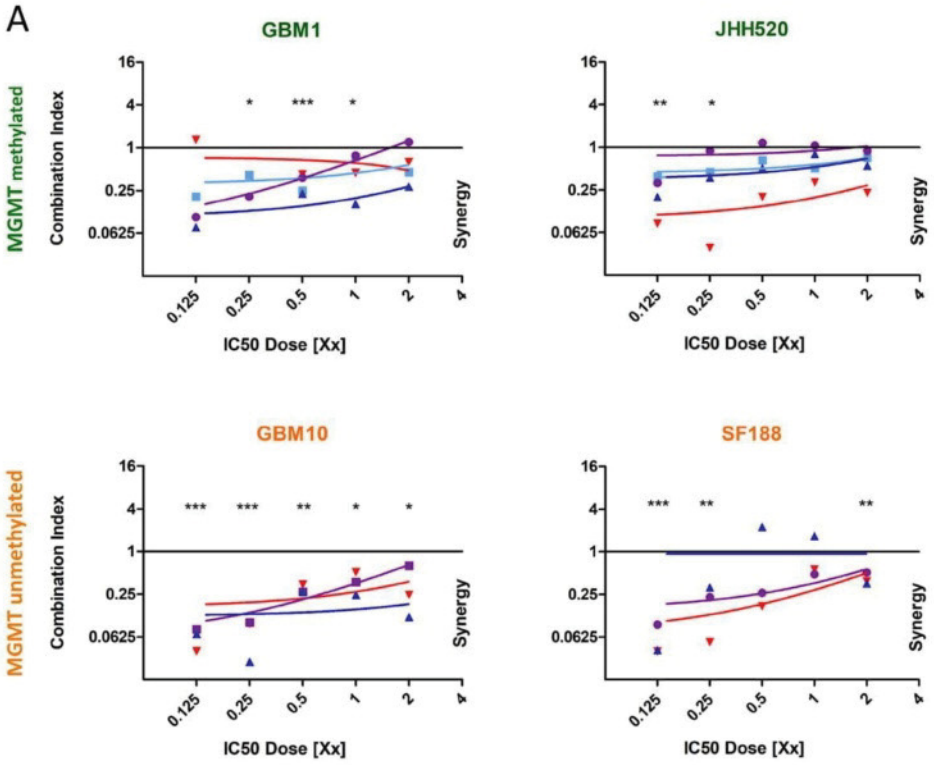
ALDH3A1 inhibition reduces *in vitro* clonogenicity and the expression of stem cell markers

To reveal effect of ALDH3A1 knock-down on glioblastoma stem-like cells, we tested the expression of several established stem cell genes in our genetically modified cell models. ALDH3A1 GBM10 knock-down cells showed reduced CD133 protein levels, whereas in GBM1 and SF188 we noticed a tendency of reduced C133 expression (Figure 4B). Of note, in cell lines when blocking ALDH3A1 we observed a significant decrease in mRNA expression of *Nestin* and *Sox2* (Figure 4C) as compared to control cells. Additionally, cells with blocked ALDH3A1 caused a strong significantly reduced the total sphere formation capacity (Figure 5).

DISCUSSION

Current therapeutic options for glioblastoma patients result in unsatisfying clinical outcomes. In particular, novel treatment options are highly needed for patients suffering from glioblastomas without *MGMT* promoter methylation who show limited benefit from TMZ chemotherapy. Our findings suggest that the enzyme ALDH3A1 might act as a therapeutic target whose inhibition sensitizes glioma cells to TMZ. Of note, ALDH3A1 blockade increased TMZ sensitivity independently of the *MGMT* promoter methylation status. Moreover, we identified Wnt signaling as an upstream regulator of this mechanism by showing that targeting Wnt pathway activity down-regulates the expression of ALDH3A1. This is of interest as Wnt signaling emerges as a therapeutic target in glioma stem-like cells [13]. Of note, in SF188, the only pediatric GBM model in our study, we observed significant differences in ALDH3A1 mRNA and protein levels in the context of drug treatment experiments. We speculate this may be a consequence of various post-transcriptional and epigenetic regulations as well as a possible negative protein-to-transcription feedback loop as previously described in large-scale glioblastoma datasets [18].

In addition to confirming recent findings that Wnt signaling promotes chemoresistance of glioblastoma cells [19], our data suggests that a possible mechanism



Gene Symbol	P value	FC (abs)	Regulation
ALDH3A1	1.46×10^{-6}	1.54	↓
CDKN1C	0.0105	1.48	↓
DLL1	0.0035	1.43	↓
PRKCQ	4.67×10^{-4}	1.37	↑
SFRP2	0.0468	1.28	↓
HIST1H1E	0.0033	1.28	↓
HIST2H2AC	0.0022	1.34	↓
HIST1H2BD	0.0046	1.23	↓
HIST1H2AL/M	0.0019	1.28	↓

Figure 1: LGK974 acts synergistically in combination with TMZ. The x-axis represents different multiples of the detected IC_{50} dose for each drug and cell line. The axis of ordinates demonstrates the calculated combination index. The combination index is calculated based on the median-effect equation, taking each value from TMZ single treatment, LGK974 single treatment and combination of both treatments for one specific dose into account. In one experiment, the combination index is calculated for five different doses and represented by one colored line. If the combination index is less than 1, both treatments act synergistically. If it is equal 1, both treatments act additively. If the combination index is more than 1, the effects are antagonistic. (**A**) LGK974 acts synergistically with TMZ in cell lines with methylated (green labeled GBM1 and JHH520) ($n = 4$ independent experiments) and unmethylated *MGMT* promoter (orange labeled GBM10 and SF188) ($n = 3$ independent experiments). (**B**) 2175 genes are significantly deregulated when combining LGK974 and TMZ treatment in GBM1 cells. Each treated group (TMZ alone, LGK974 alone, TMZ plus LGK974) was compared to the DMSO-treated control group ($n = 4$ independent experiments). 1800 genes were differentially expressed in the TMZ-treated group, 954 genes in the LGK974-treated group and 177 genes were expressed in all treated groups compared to DMSO control. (**C**) The nine genes with highest fold-change in the combinatory treatment group. Abbr.: D: DMSO; T: TMZ; L: LGK974.$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. additive effect (unpaired student *t*-test).

of this association is the down regulation of ALDH3A1 expression in response to pathway inhibition. We chose ALDH3A1 as our target of interest since family members of the ALDH group are known to detoxify reactive aldehydes caused by treatment with alkylating chemotherapeutics [20, 21].

We consistently observed most synergistic anti-growth effects in lower dosages of TMZ and LGK974. This observation might be explained by the dose-effect curve for LGK974 indicating high effectiveness in low dosages. Given the particularly high synergistic effect observed when treating the cells with low drug

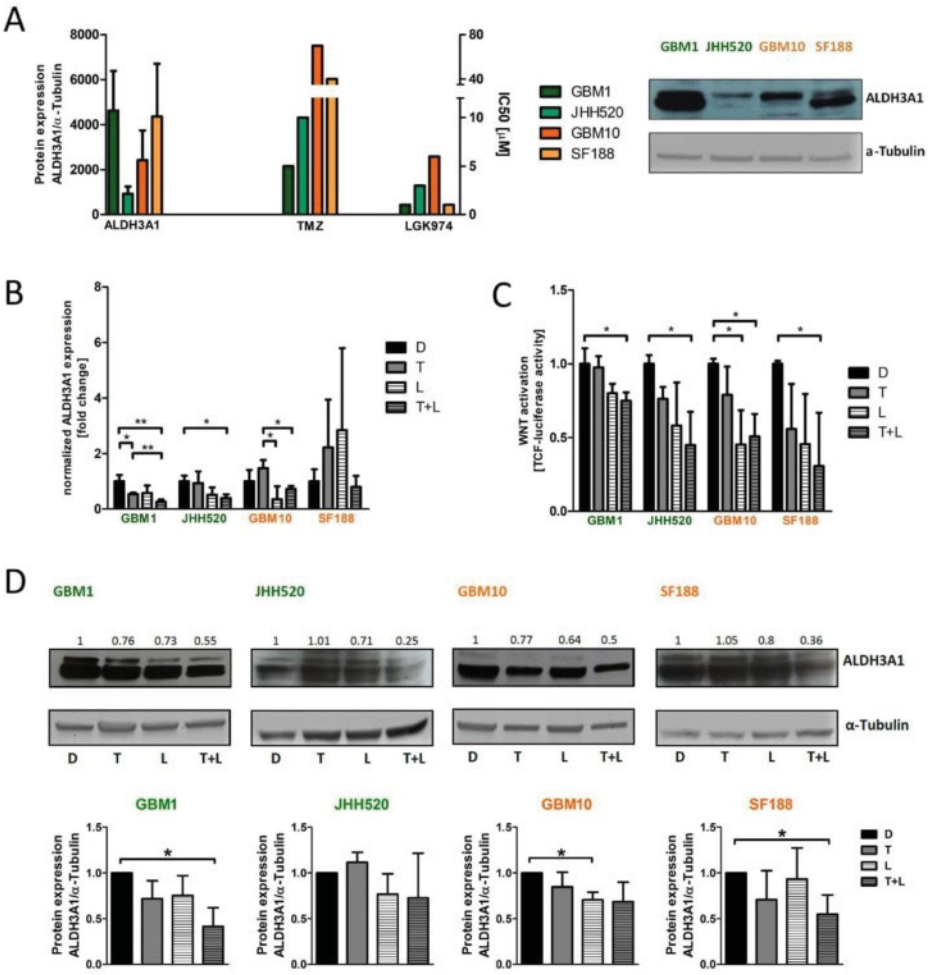


Figure 2: ALDH3A1 mRNA and protein expression is down-regulated in glioma cells treated with LGK974 and TMZ. (**A**) Comparison between basal protein levels of ALDH3A1 (left) and IC_{50} dosages of TMZ and LGK974 (right) for all four cell lines. One blot of basal ALDH3A1 expression is shown (n = 3 individual experiments). (**B**) *ALDH3A1* mRNA expression in the four glioma cell lines. Note that *ALDH3A1* mRNA expression is significantly down-regulated in the *MGMT* promoter-methylated cell lines GBM1 and JHH520 in the LGK974 plus TMZ treatment group (n = 3 independent experiments). (**C**) Wnt pathway activity assessed by T cell factor (TCF) luciferase reporters. Wnt activity is significantly reduced upon combined treatment with TMZ and LGK974 in all cell lines (n = 3 independent experiments). (**D**) Protein expression of ALDH3A1 in the investigated four glioma cell lines upon treatment. Quantification of blots from n = 3 independent experiments reveal, ALDH3A1 protein expression is down-regulated in the LGK974 plus TMZ treatment group in GBM1 and SF188. GBM10 showed significant suppression of ALDH3A1 when treated with LGK974 alone, JHH520 did not reduce ALDH3A1 expression. . MGMT methylated cell lines presented in green, MGMT unmethylated cell lines presented in orange. Data are presented as mean ± standard deviation (SD). $^{*}p < 0.05$ (unpaired student t-test). D, DMSO; T, TMZ; L, LGK974.

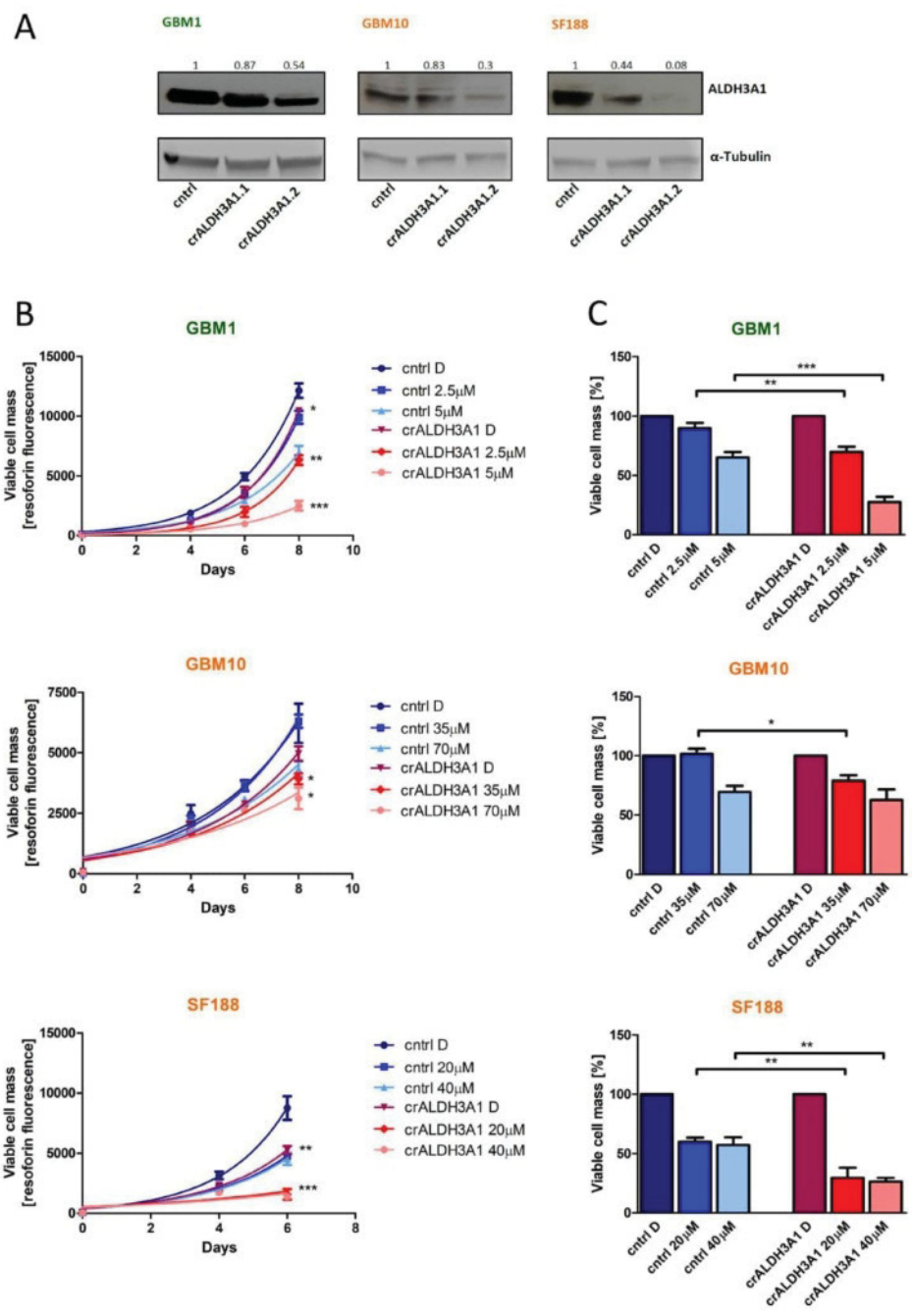


Figure 3: *ALDH3A1* knock-down reduces cell viability and sensitizes cells to TMZ. (**A**) Two individual knock-downs of ALDH3A1 were established in three cell lines. α-Tubulin served as gel loading control. (**B**) *ALDH3A1* knock-down (crALDH3A1.2) and control cells were treated with two different concentrations of TMZ, based on the IC_{50} of the control cells ($1 \times IC_{50}$, $0.5 \times IC_{50}$). Cell viability was measured at day 0, 4, 6 (and 8 in slowly growing cells). Statistical evaluation was performed for *ALDH3A1* knock-down cells compared to control cells treated with DMSO or the same concentration of TMZ. (**C**) Cell viability of the DMSO-treated cells of either ALDH3A1 knock-down or control cells is normalized to 100%. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (unpaired student *t*-test). D: DMSO.

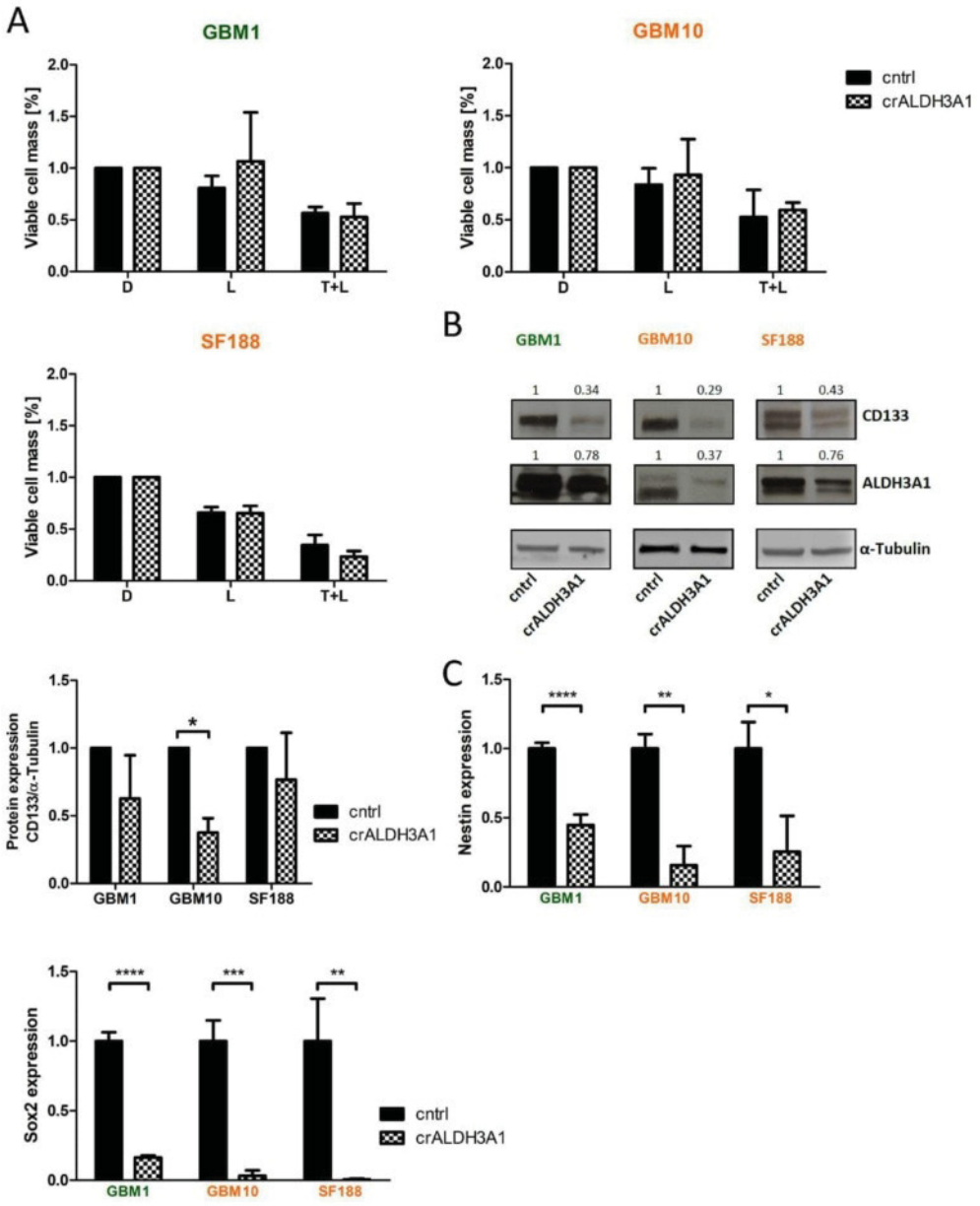


Figure 4: *ALDH3A1* knock-down is not effected by Wnt-inhibition and shows reduced stemness properties. (**A**) *ALDH3A1* knock-down (crALDH3A1.2) cells show similar response to LGK974 and combination with TMZ treatment as control cells. Cell viability was measured at day 6. Cell viability of the DMSO-treated cells of either ALDH3A1 knock-down or control cells is normalized to 100% ($n = 3$ independent experiments). (**B**) Protein expression of CD133 in GBM10 is strongly reduced when targeting ALDH3A1 as compared to control cells. In GBM1 and SF188 a tendency of CD133 was noticed. Quantifications of three independent experiments are shown. (**C**) mRNA expression of stemness factors Nestin and Sox2 is significantly down-regulated in ALDH3A1 knock-down cells as compared to control cells ($n = 3$ independent experiments). MGMT methylated cell lines presented in green, MGMT unmethylated cell lines presented in orange. Data are presented as mean ± standard deviation (SD). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ (unpaired student *t*-test). D: DMSO; T: TMZ; L: LGK974.

concentrations, we hypothesize that adverse effects could be minimized and thereby favoring clinical applicability.

Additionally, our functional data suggest the utility of high ALDH3A1 expression as a putative diagnostic marker for stemness in glioma as indicated by reduced expression of CD133, Nestin and Sox2 as well as diminished clonogenicity in response to ALDH3A1 inhibition. Further correlative investigations in clinical datasets and *in vivo* models are needed to verify this hypothesis. Moreover, decreased overall cell viability following reduced ALDH3A1 expression could be due to the depletion of glioma cells with stem-like properties. However, additional functional studies are needed to comprehensively decipher the mechanistic background of

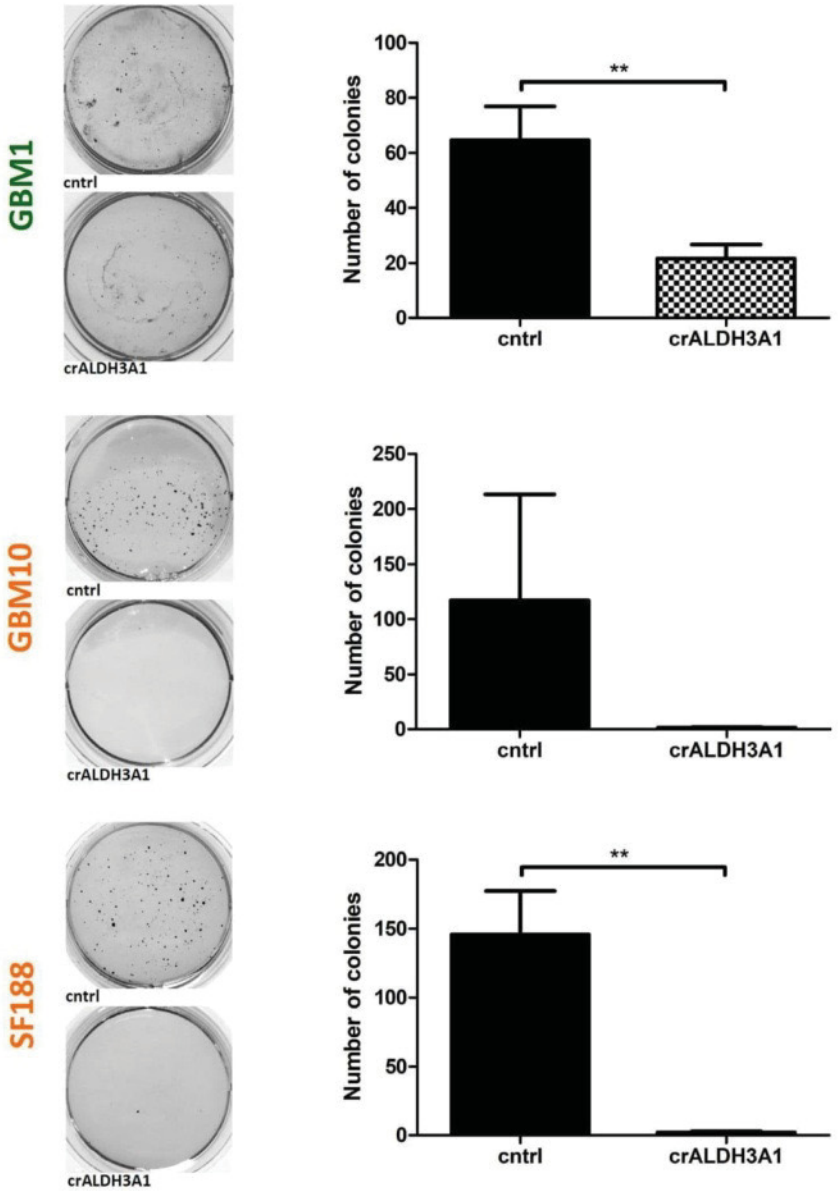


Figure 5: Clonogenicity is reduced in *ALDH3A1* knock-down cells. Representative pictures of NBT stained colonies and quantifications of three colony forming assays are shown ($n = 3$ independent experiments). $^{**}p < 0.01$.

interaction between stemness and cell growth in relation to ALDH3A1 expression and Wnt signaling activation.

The data reported in the present study is in concordance with observations in other solid tumors. In both preclinical and clinical studies it has been reported that high levels of ALDH3A1 promote chemoresistance towards various common anti-cancer drugs and could be correlated with poor clinical prognosis [15, 22–26]. Moreover, reports on other tumors outside the central nervous system suggest a positive correlation of ALDH3A1 expression and β-catenin signaling levels [26–29]. In head and neck squamous cell carcinoma, activation of ALDH3A1 increases chemoresistance against cisplatin whereas combining cisplatin with an ALDH inhibitor results in more pronounced cell viability reduction than treatment with each compound alone [30]. Other members of the ALDH family were already proposed to mediate therapy resistance in glioblastoma. ALDH1A3 was shown to increase resistance towards γ-radiation and to be up-regulated in high grade gliomas, whereas ALDH1A3 promoter-methylation correlated with longer survival time [31, 32]. Schäfer *et al.* identified ALDH1A1 to induce resistance towards TMZ in glioblastoma *in vitro* [33]. Importantly, we could not find any correlation between spontaneous ALDH3A1 mRNA expression and resistance levels to TMZ, suggesting that sensitivity to TMZ is only partly mediated through ALDH3A1. However, we provide evidence for ALDH3A1 inhibition to function as sensitizer to the glioblastoma standard of care chemotherapeutic agent.

The porcupine inhibitor LGK974 reduces Wnt signaling and decreased expression of ALDH3A1 mRNA and protein in glioma cells, thereby increasing their susceptibility to TMZ treatment. Regulation of *ALDH3A1* transcription by Wnt pathway activity may be mediated by increased TCF/LEF binding to the *ALDH3A1* gene promoter harboring TCF/LEF binding motifs (Supplementary Figure 3) [34]. However, this hypothesis requires further experimental proof. By comparing the results from wildtype cells with those from cells with genetically inhibited ALDH3A1 expression, it seems as if the ALDH3A1 knock-down is more efficient in reducing cell viability than pharmacological Wnt-inhibition. This result might be explained by the fact that LGK974 is a porcupine inhibitor and hence possibly not only affecting Wnt signaling but also further off-targets. Nevertheless, our results also suggest that the observed suppression of cellular growth upon Wnt blockade is mediated by suppression of ALDH3A1 for the most part, since LGK974 treatment has no effect on ALDH3A1 knock-down cells. Besides, we could not find a correlation between Wnt activation and ALDH3A1 expression in our cell lines, indicating that ALDH3A1 might not only be regulated through Wnt/β-catenin signaling but ALDH3A1 expression is also affected by other cellular dynamics.

Our findings also suggest that ALDH3A1 may be a promising therapeutic target for glioblastomas resistant to the standard of care treatment. The potential of ALDH3A1 as a therapeutic target with low adverse effects has been shown in *ALDH3A1* knockout mice [35]. Except for eye cataracts, a consequence of destruction of fiber cells that are dependent on ALDH3A1 to minimize oxygen damage, *ALDH3A1* knockout mice showed equal survival and growth as control animals. Of note, specific inhibitors of ALDH3A1 have been developed and shown to enhance the sensitivity of cancer cells to the alkylating agent cyclophosphamide [20, 22]. Our data also indicate that ALDH3A1 may regulate TMZ sensitivity in glioblastoma cells independently of the *MGMT* promoter methylation status. However, preclinical *in vivo* studies with drugs targeting ALDH3A1 are compulsory to further substantiate our *in vitro* findings and translate them into novel targeted treatment approach. Whereas ALDH expression serves as biological marker in solid tumors, we could not verify any prognostic value for ALDH3A1 in glioblastoma by searching several data bases (The Cancer Genome Atlas (TCGA) https://cancergenome.nih.gov/, The Cancer Imaging Archive (TCIA) [36], Murat *et al.* [37], Reifenberger *et al.* [38]).

In summary, our results reveal that ALDH3A1 expression in glioma cells can be modulated by Wnt pathway inhibition. Furthermore, we show that down-regulation of ALDH3A1 increases TMZ sensitivity and reduces stemness features in glioma cells *in vitro* independent of the *MGMT* promoter methylation status. These findings may have clinical significance by suggesting inhibition of ALDH3A1 as a potential strategy for increasing TMZ efficacy and particularly targeting the highly malignant subpopulation of stem-like glioma cells.

MATERIALS AND METHODS

Cell culture, pharmacological and radiation treatment

All four glioblastoma cell lines (GBM1, GBM10, JHH520, SF188) were cultivated in neurosphere medium containing 70% serum-free Dulbecco modified Eagle medium and 30% F12 (both Gibco BRL, Eggenstein, Germany), supplemented with 2% B27 (Gibco BRL), 20 ng/ml bovine fibroblast growth factor (Peprotech, Rocky Hill, NJ), 20 ng/ml human epidermal growth factor (Peprotech), 5ug/ml heparin (Sigma-Aldrich, St Louis, MO) and 1% Anti-Anti Penicillin Steptomycin Fungizone® mixture (Gibco). All cell lines were cultivated under standard cell culture conditions at 37° C temperature and 5% carbon dioxide. They were regularly tested for the absence of mycoplasma contamination using the PCR-based Mycoplasma Test Kit I/C from Promokine (Heidelberg, Germany). GBM1 was generously provided

by A. Vescovi (Milan, Italy); GBM10 was provided by C. Eberhart [39]; JHH520 was provided by G. Riggins; the pediatric GBM cell line SF188 was provided by E. Raabe [40] (all Baltimore, USA). LGK974 (Selleckchem, Houston, USA) and temozolomide (Sigma-Aldrich, St. Louis, USA) were diluted in DMSO (Sigma- Aldrich) and stored in −20° C/−80° C. Both drugs were diluted in medium and added after each cell passage. Cells were passaged every second day and medium containing fresh drug was substituted. For radiation treatment, cells were exposed once to γ-radiation using a Gulmay RS225 X-ray system from X-Strahl (Camberley, UK).

MGMT promoter methylation analysis

The methylation status of the *MGMT* promoter was determined by methylation-specific PCR as reported before [41]. The glioma cell line A172 served a positive control for *MGMT* promoter methylation whereas DNA extracted from peripheral blood leukocytes served as a negative control.

Cell viability assays

Triplicates of 2000–5000 cells per well (depending on cell line) were plated on laminin-coated 96-well-plates in 100ul media for 6 days. The definition of maximal inhibitory concentration (IC50) of applied stimuli (LGK974, TMZ, γ-radiation) was performed through quantification of reduction of cellular viability using the CellTiter Blue™ Cell Viability Assay (Promega, Fitchburg, USA) as previously reported [14]. Fluorescence was measured after 2 hours incubation time using the Tecan Safire 2 Multiplate Reader (Tecan, Männedorf, Switzerland) at 560ex/590em. For IC50 definition we compared the effect on day 6 after treatment while changing medium supplemented with fresh drug every 2nd day. For analyzing combinatory effects, cells were treated with 5 different concentrations (0.25×, 0.5×, 1×, 2× and 0.125× or 4× IC50) of each drug or γ-radiation as mono or combination therapy. Viability measurements where performed as described above for the IC50 experiments. The combination index (CI) was calculated as described before [42] using the program CompuSyn (ComboSyn Inc., Paramus, NJ. 07652 USA) [16]. A CI < 1 refers to a synergistic, CI = 1 to an additive and CI > 1 to an antagonistic effect.

Gene knock-down in glioblastoma cells using Crispr/Cas9- and shRNA-based approaches

Lentiviral particles of the third generation were generated for infecting cells as reported [43]. Cells with stable integration were selected using 2 μg/mL puromycin (Sigma-Aldrich). For *ALDH3A1* gene knock-down we used the lentiCRISPRv2 plasmid (Addgene plasmid # 52961) [44]. Oligonucleotides for guide RNAs were designed using the CRISPR design tool provided by the Zhang lab (http://crispr.mit.edu/). Oligonucleotides targeting GFP were used as a control. β-catenin knock-down was achieved by cloning shRNA into a pLKO.1 vector (Addgene plasmid # 1248) [45]. The RNA targeting sequences used are provided in the Supplementary Table 1B.

Quantitative PCR and microarray-based gene expression analysis

RNA extraction (RNeasy Mini Kit, Qiagen) and cDNA synthesis (using M-MLV reverse transcriptase, Promega) were performed according to the manufacturer´s instructions. For the qPCR SsoAdvanced SYBR Green Supermix (BioRad) was used in a CFX Connect Thermocycler (BioRad), and the reaction was normalized to the housekeeping gene β2-microglobulin employing the $\Delta\Delta C_t$ method. Primer sequences are provided in Supplementary Table 1A. Transcriptome-wide expression analysis was performed on GBM1 cells after 72 h under drug treatment using Affymetrix GeneChip PrimeView Human Gene Expression Arrays (Affymetrix, Santa Clara, USA). Total RNA preparations were checked for RNA integrity by using the Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, USA). All samples in this study showed high quality RNA Integrity Numbers (RIN = 10). RNA was further analysed by photometric Nanodrop measurement and quantified by fluorometric Qubit RNA assays (Life Technologies). Synthesis of cDNA and subsequent biotin labelling of cRNA was performed according to the manufacturers´ protocol (3′ IVT Plus Kit; Affymetrix Inc.). Briefly, 100 ng of total RNA were converted to cDNA, followed by *in vitro* transcription and biotin labelling of cRNA. After fragmentation, labelled cRNA was hybridized to Affymetrix PrimeView Human Gene Expression Microarrays for 16 h at 45° C, stained by streptavidin/phycoerythrin conjugate and scanned as described in the manufacturers´ protocol. Data analyses on Affymetrix CEL files were conducted with GeneSpring GX software (Vers. 12.5; Agilent Technologies). To further improve signal-to-noise ratio, a given probeset had to be expressed above background (i.e. fluorescence signal of a probe set was detected within the 20th and 100th percentiles of the raw signal distribution of a given array) in all four replicates in at least one of two, or both conditions to be subsequently analyzed in pairwise comparisons.

Western blot analyses

Total proteins were extracted from glioma cells using RIPA Buffer as reported. Protein concentrations were determined in a Tecan Safire 2 Multiplate reader (Tecan) using the DC Protein Assay Kit (Biorad) due to the manufacturer´s instructions. Primary antibodies

(ALDH3A1, 1/1000, abcam # ab76976; β-catenin, 1/1000, BD # 610153; α-Tubulin, 1/10000, Sigma # T9026; CD133, 1/100, Miltenyi Biotec # W6B3C1) were incubated overnight at 4°. Secondary antibodies (goat-anti-rabbit, IRDye800CW LI-COR # 926-32211; goat-anti-mouse, IRDye680RD LI-COR # 926-68070; goat anti-rabbit-HRP, Jackson Immuno Research # 111-035-144; all 1/10000) were incubated 1h at room temperature. All antibodies were diluted in blocking solution containing 5% milk powder in Tris-buffered saline with Tween20 (TBST). Signals were detected using a film based system by applying Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) or a luminescence based system in a LI-COR Odyssey CLx Imager (LI-COR). Densitometry was done using supplied software from LI-COR or ImageJ software.

Reporter assay for measurement of Wnt/CTNNB1 activity

To detect canonical Wnt pathway activity we installed a stable transfection of our cells with a reporter construct containing seven TCF binding sides followed by a firefly luciferase cassette, as previously described [14]. Transfected cells were selected using 2 µg/ml puromycin (Sigma-Aldrich). For each measurement, cells were harvested and washed in PBS. According to the manufacturer's protocol the cells were lysed in Lysis Solution (Life Technologies # T1003). Luminescence readout was performed at 490 nm emission wavelength on a TriStar LB941 luminometer (Berthold Technologies, Bad Wildbach, Germany) and normalized to ß-galactosidase activity.

Clonogenicity assay sigma-aldrich

For assessing clonogenic capacity of our cell lines we performed a colony formation assay in soft agarose as described previously [14]. Six-well plated were coated with a bottom layer consisting of 1.5 ml of 1% agarose (Life Technologies) and neurosphere media. On top a 2 ml layer consisting of 0.6% agarose containing 5000 cell/well was plated. It was covered with additional media (2 ml). After 3 weeks, 1 mg/ml 4-Nitro blue tetrazolium chloride (NBT) solution (Sigma-Aldrich) was added to stain the colonies overnight at 37° C. The experiments were quantified using Clono Counter software [46].

Analysis of human tissue samples and published glioblastoma expression datasets

For prognostic associations of *ALDH3A1* mRNA expression in glioblastoma patients, we retrieved publically available data sets from *Reifenberger et al.* (GEO accession no. GSE53733).

Statistical analyses

All cell biological experiments were done in at least three independent experiments and results are shown as mean ± standard deviation (SD). An unpaired student *t* test was performed with Prism version 4 (GraphPad Software Inc, La Jolla, CA) to calculate statistical significance and $p < 0.05$ was considered as significant. The correlation coefficient was also calculated using Prism. For transcriptome-wide expression analysis, probes within each probeset were summarized by Robust Multi-array Average (RMA) after quantile normalization of probe level signal intensities across all samples to reduce inter-array variability [47]. Input data pre-processing was concluded by baseline transformation to the median of all samples. To further improve signal-to-noise ratio, a given probeset had to be expressed above background (i.e. fluorescence signal of a probeset was detected within the 20th and 100th percentiles of the raw signal distribution of a given array) in all four replicates in at least one of two, or both conditions to be subsequently analysed in pairwise comparisons.

Abbreviations

ALDH3A1: aldehyddehydrogenase 3A1; GSC: glioma stem cell; IC_{50}: half maximal inhibitory concentration; IDH: isocitrate dehydrogenase; MGMT: O-6-methylguanine-DNA-methyltransferase; TCF: transcription factor; TMZ: temozolomide; Wnt: wingless.

Author contributions

Suwala AK has contributed to study design, collecting, analyzing and interpretation of the data and writing of the manuscript. Koch K has contributed to collecting, analyzing and interpretation of the data. Herrera Rios D, Aretz P and Uhlmann C have contributed to collecting and analyzing data. Ogorek I has contributed to collecting data. Felsberg J has contributed to collecting, analyzing and interpretation of the data. Reifenberger G has contributed to collecting data, interpretation of the data and writing the manuscript. Köhrer K has contributed to collecting and analyzing data. Deenen R has contributed to collecting, analyzing and interpretation of the data. Steiger HJ has contributed to data analysis. Kahlert UD has contributed to study design, interpretation of the data and writing the manuscript. Maciaczyk J has contributed to study design, interpretation of the data and writing the manuscript.

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CONFLICTS OF INTEREST

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REFERENCES

1. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, et al, and European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups, and National Cancer Institute of Canada Clinical Trials Group. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med. 2005; 352:987–96. https://doi.org/10.1056/NEJMoa043330.

2. Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, Ludwin SK, Allgeier A, Fisher B, Belanger K, Hau P, Brandes AA, Gijtenbeek J, et al, and European Organisation for Research and Treatment of Cancer Brain Tumour and Radiation Oncology Groups, and National Cancer Institute of Canada Clinical Trials Group. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol. 2009; 10:459–66. https://doi.org/10.1016/S1470-2045(09)70025-7.

3. Wick W, Weller M, van den Bent M, Sanson M, Weiler M, von Deimling A, Plass C, Hegi M, Platten M, Reifenberger G. MGMT testing—the challenges for biomarker-based glioma treatment. Nat Rev Neurol. 2014; 10:372–85. https://doi.org/10.1038/nrneurol.2014.100.

4. Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, Kros JM, Hainfellner JA, Mason W, Mariani L, Bromberg JE, Hau P, Mirimanoff RO, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med. 2005; 352:997–1003. https://doi.org/10.1056/NEJMoa043331.

5. Lu-Emerson C, Duda DG, Emblem KE, Taylor JW, Gerstner ER, Loeffler JS, Batchelor TT, Jain RK. Lessons from anti-vascular endothelial growth factor and anti-vascular endothelial growth factor receptor trials in patients with glioblastoma. J Clin Oncol. 2015; 33:1197–213. https://doi.org/10.1200/JCO.2014.55.9575.

6. Salmaggi A, Boiardi A, Gelati M, Russo A, Calatozzolo C, Ciusani E, Sciacca FL, Ottolina A, Parati EA, La Porta C, Alessandri G, Marras C, Croci D, De Rossi M. Glioblastoma-derived tumorospheres identify a population of tumor stem-like cells with angiogenic potential and enhanced multidrug resistance phenotype. Glia. 2006; 54:850–60. https://doi.org/10.1002/glia.20414.

7. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD, Rich JN. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature. 2006; 444:756–60. https://doi.org/10.1038/nature05236.

8. Lathia JD, Mack SC, Mulkearns-Hubert EE, Valentim CL, Rich JN. Cancer stem cells in glioblastoma. Genes Dev. 2015; 29:1203–17. https://doi.org/10.1101/gad.261982.115.

9. Liu J, Pan S, Hsieh MH, Ng N, Sun F, Wang T, Kasibhatla S, Schuller AG, Li AG, Cheng D, Li J, Tompkins C, Pferdekamper A, et al. Targeting Wnt-driven cancer through the inhibition of Porcupine by LGK974. Proc Natl Acad Sci USA. 2013; 110:20224–29. https://doi.org/10.1073/pnas.1314239110.

10. Emami KH, Nguyen C, Ma H, Kim DH, Jeong KW, Eguchi M, Moon RT, Teo JL, Oh SW, Kim HY, Moon SH, Ha JR, Kahn M. A small molecule inhibitor of beta-catenin/CREB-binding protein transcription [corrected]. Proc Natl Acad Sci USA. 2004; 101:12682–87. https://doi.org/10.1073/pnas.0404875101.

11. Lenz HJ, Kahn M. Safely targeting cancer stem cells via selective catenin coactivator antagonism. Cancer Sci. 2014; 105:1087–92. https://doi.org/10.1111/cas.12471.

12. Gang EJ, Hsieh YT, Pham J, Zhao Y, Nguyen C, Huantes S, Park E, Naing K, Klemm L, Swaminathan S, Conway EM, Pelus LM, Crispino J, et al. Small-molecule inhibition of CBP/catenin interactions eliminates drug-resistant clones in acute lymphoblastic leukemia. Oncogene. 2014; 33:2169–78. https://doi.org/10.1038/onc.2013.169.

13. Suwala AK, Hanaford A, Kahlert UD, Maciaczyk J. Clipping the Wings of Glioblastoma: Modulation of WNT as a Novel Therapeutic Strategy. J Neuropathol Exp Neurol. 2016; 75:388–96. https://doi.org/10.1093/jnen/nlw013.

14. Kahlert UD, Suwala AK, Koch K, Natsumeda M, Orr BA, Hayashi M, Maciaczyk J, Eberhart CG. Pharmacologic Wnt Inhibition Reduces Proliferation, Survival, and Clonogenicity of Glioblastoma Cells. J Neuropathol Exp Neurol. 2015; 74:889–900. https://doi.org/10.1097/NEN.0000000000000227.

15. Sládek NE, Kollander R, Sreerama L, Kiang DT. Cellular levels of aldehyde dehydrogenases (ALDH1A1 and ALDH3A1) as predictors of therapeutic responses to cyclophosphamide-based chemotherapy of breast cancer: a retrospective study. Rational individualization of oxazaphosphorine-based cancer chemotherapeutic regimens. Cancer Chemother Pharmacol. 2002; 49:309–21. https://doi.org/10.1007/s00280-001-0412-4.

16. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev. 2006; 58:621–81. https://doi.org/10.1124/pr.58.3.10.

17. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul. 1984; 22:27–55. https://doi.org/10.1016/0065-2571(84)90007-4.

18. Brennan CW, Verhaak RG, McKenna A, Campos B, Noushmehr H, Salama SR, Zheng S, Chakravarty D, Sanborn JZ, Berman SH, Beroukhim R, Bernard B, Wu CJ, et al, and TCGA Research Network. The somatic genomic landscape of glioblastoma. Cell. 2013; 155:462–77. https://doi.org/10.1016/j.cell.2013.09.034.

19. Wickström M, Dyberg C, Milosevic J, Einvik C, Calero R, Sveinbjörnsson B, Sandén E, Darabi A, Siesjö P, Kool M, Kogner P, Baryawno N, Johnsen JI. Wnt/β-catenin pathway regulates MGMT gene expression in cancer and inhibition of Wnt signalling prevents chemoresistance. Nat Commun. 2015; 6:8904. https://doi.org/10.1038/ncomms9904.

20. Parajuli B, Georgiadis TM, Fishel ML, Hurley TD. Development of selective inhibitors for human aldehyde dehydrogenase 3A1 (ALDH3A1) for the enhancement of cyclophosphamide cytotoxicity. ChemBioChem. 2014; 15:701–12. https://doi.org/10.1002/cbic.201300625.

21. Pors K, Moreb JS. Aldehyde dehydrogenases in cancer: an opportunity for biomarker and drug development? Drug Discov Today. 2014; 19:1953–63. https://doi.org/10.1016/j.drudis.2014.09.009.

22. Parajuli B, Fishel ML, Hurley TD. Selective ALDH3A1 inhibition by benzimidazole analogues increase mafosfamide sensitivity in cancer cells. J Med Chem. 2014; 57:449–61. https://doi.org/10.1021/jm401508p.

23. Kuo SH, Yang SY, You SL, Lien HC, Lin CH, Lin PH, Huang CS. Polymorphisms of ESR1, UGT1A1, HCN1, MAP3K1 and CYP2B6 are associated with the prognosis of hormone receptor-positive early breast cancer. Oncotarget. 2017; 8:20925–38. https://doi.org/10.18632/oncotarget.14995.

24. Hu G, Chong RA, Yang Q, Wei Y, Blanco MA, Li F, Reiss M, Au JL, Haffty BG, Kang Y. MTDH activation by 8q22 genomic gain promotes chemoresistance and metastasis of poor-prognosis breast cancer. Cancer Cell. 2009; 15:9–20. https://doi.org/10.1016/j.ccr.2008.11.013.

25. Yan J, De Melo J, Cutz JC, Aziz T, Tang D. Aldehyde dehydrogenase 3A1 associates with prostate tumorigenesis. Br J Cancer. 2014; 110:2593–603. https://doi.org/10.1038/bjc.2014.201.

26. Voulgaridou GP, Kiziridou M, Mantso T, Chlichlia K, Galanis A, Koukourakis MI, Franco R, Panayiotidis MI, Pappa A. Aldehyde dehydrogenase 3A1 promotes multi-modality resistance and alters gene expression profile in human breast adenocarcinoma MCF-7 cells. Int J Biochem Cell Biol. 2016; 77:120–28. https://doi.org/10.1016/j.biocel.2016.06.004.

27. Zhang F, Song C, Ma Y, Tang L, Xu Y, Wang H. Effect of fibroblasts on breast cancer cell mammosphere formation and regulation of stem cell-related gene expression. Int J Mol Med. 2011; 28:365–71. https://doi.org/10.3892/ijmm.2011.700.

28. Calderaro J, Nault JC, Bioulac-Sage P, Laurent A, Blanc JF, Decaens T, Zucman-Rossi J. ALDH3A1 is overexpressed in a subset of hepatocellular carcinoma characterised by activation of the Wnt/ß-catenin pathway. Virchows Arch. 2014; 464:53–60. https://doi.org/10.1007/s00428-013-1515-0.

29. Alix-Panabières C, Cayrefourcq L, Mazard T, Maudelonde T, Assenat E, Assou S. Molecular Portrait of Metastasis-Competent Circulating Tumor Cells in Colon Cancer Reveals the Crucial Role of Genes Regulating Energy Metabolism and DNA Repair. Clin Chem. 2017; 63:700–13. https://doi.org/10.1373/clinchem.2016.263582.

30. Kim J, Shin JH, Chen CH, Cruz L, Farnebo L, Yang J, Borges P, Kang G, Mochly-Rosen D, Sunwoo JB. Targeting aldehyde dehydrogenase activity in head and neck squamous cell carcinoma with a novel small molecule inhibitor. Oncotarget. 2017; 8:52345–56. https://doi.org/10.18632/oncotarget.17017.

31. Zhang W, Yan W, You G, Bao Z, Wang Y, Liu Y, You Y, Jiang T. Genome-wide DNA methylation profiling identifies ALDH1A3 promoter methylation as a prognostic predictor in G-CIMP- primary glioblastoma. Cancer Lett. 2013; 328:120–25. https://doi.org/10.1016/j.canlet.2012.08.033.

32. Mao P, Joshi K, Li J, Kim SH, Li P, Santana-Santos L, Luthra S, Chandran UR, Benos PV, Smith L, Wang M, Hu B, Cheng SY, et al. Mesenchymal glioma stem cells are maintained by activated glycolytic metabolism involving aldehyde dehydrogenase 1A3. Proc Natl Acad Sci USA. 2013; 110:8644–49. https://doi.org/10.1073/pnas.1221478110.

33. Schäfer A, Teufel J, Ringel F, Bettstetter M, Hoepner I, Rasper M, Gempt J, Koeritzer J, Schmidt-Graf F, Meyer B, Beier CP, Schlegel J. Aldehyde dehydrogenase 1A1—a new mediator of resistance to temozolomide in glioblastoma. Neuro-oncol. 2012; 14:1452–64. https://doi.org/10.1093/neuonc/nos270.

34. Cadigan KM, Waterman ML. TCF/LEFs and Wnt Signaling in the Nucleus. Cold Spring Harb Perspect Biol. 2012; 4:a007906. https://doi.org/10.1101/cshperspect.a007906.

35. Lassen N, Bateman JB, Estey T, Kuszak JR, Nees DW, Piatigorsky J, Duester G, Day BJ, Huang J, Hines LM, Vasiliou V. Multiple and additive functions of ALDH3A1

and ALDH1A1: cataract phenotype and ocular oxidative damage in Aldh3a1(-/-)/Aldh1a1(-/-) knock-out mice. J Biol Chem. 2007; 282:25668–76. https://doi.org/10.1074/jbc.M702076200.

36. Clark K, Vendt B, Smith K, Freymann J, Kirby J, Koppel P, Moore S, Phillips S, Maffitt D, Pringle M, Tarbox L, Prior F. The Cancer Imaging Archive (TCIA): maintaining and operating a public information repository. J Digit Imaging. 2013; 26:1045–57. https://doi.org/10.1007/s10278-013-9622-7.

37. Murat A, Migliavacca E, Gorlia T, Lambiv WL, Shay T, Hamou MF, de Tribolet N, Regli L, Wick W, Kouwenhoven MC, Hainfellner JA, Heppner FL, Dietrich PY, et al. Stem cell-related "self-renewal" signature and high epidermal growth factor receptor expression associated with resistance to concomitant chemoradiotherapy in glioblastoma. J Clin Oncol. 2008; 26:3015–24. https://doi.org/10.1200/JCO.2007.15.7164.

38. Reifenberger G, Weber RG, Riehmer V, Kaulich K, Willscher E, Wirth H, Gietzelt J, Hentschel B, Westphal M, Simon M, Schackert G, Schramm J, Matschke J, et al, and German Glioma Network. Molecular characterization of long-term survivors of glioblastoma using genome- and transcriptome-wide profiling. Int J Cancer. 2014; 135:1822–31. https://doi.org/10.1002/ijc.28836.

39. Kahlert UD, Bender NO, Maciaczyk D, Bogiel T, Bar EE, Eberhart CG, Nikkhah G, Maciaczyk J. CD133/CD15 defines distinct cell subpopulations with differential *in vitro* clonogenic activity and stem cell-related gene expression profile in *in vitro* propagated glioblastoma multiforme-derived cell line with a PNET-like component. Folia Neuropathol. 2012; 50:357–68. https://doi.org/10.5114/fn.2012.32365.

40. Mao XG, Hütt-Cabezas M, Orr BA, Weingart M, Taylor I, Rajan AK, Odia Y, Kahlert U, Maciaczyk J, Nikkhah G, Eberhart CG, Raabe EH. LIN28A facilitates the transformation of human neural stem cells and promotes glioblastoma tumorigenesis through a pro-invasive genetic program. Oncotarget. 2013; 4:1050–64. https://doi.org/10.18632/oncotarget.1131.

41. Felsberg J, Rapp M, Loeser S, Fimmers R, Stummer W, Goeppert M, Steiger HJ, Friedensdorf B, Reifenberger G, Sabel MC. Prognostic significance of molecular markers and extent of resection in primary glioblastoma patients. Clin Cancer Res. 2009; 15:6683–93. https://doi.org/10.1158/1078-0432.CCR-08-2801.

42. Koch K, Hartmann R, Schröter F, Suwala AK, Maciaczyk D, Krüger AC, Willbold D, Kahlert UD, Maciaczyk J. Reciprocal regulation of the cholinic phenotype and epithelial-mesenchymal transition in glioblastoma cells. Oncotarget. 2016; 7:73414-31. https://doi.org/10.18632/oncotarget.12337.

43. Kahlert UD, Suwala AK, Raabe EH, Siebzehnrubl FA, Suarez MJ, Orr BA, Bar EE, Maciaczyk J, Eberhart CG. ZEB1 Promotes Invasion in Human Fetal Neural Stem Cells and Hypoxic Glioma Neurospheres. Brain Pathol. 2015; 25:724–32. https://doi.org/10.1111/bpa.12240.

44. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. Nat Methods. 2014; 11:783–84. https://doi.org/10.1038/nmeth.3047.

45. Kahlert UD, Maciaczyk D, Doostkam S, Orr BA, Simons B, Bogiel T, Reithmeier T, Prinz M, Schubert J, Niedermann G, Brabletz T, Eberhart CG, Nikkhah G, Maciaczyk J. Activation of canonical WNT/β-catenin signaling enhances *in vitro* motility of glioblastoma cells by activation of ZEB1 and other activators of epithelial-to-mesenchymal transition. Cancer Lett. 2012; 325:42–53. https://doi.org/10.1016/j.canlet.2012.05.024.

46. Niyazi M, Niyazi I, Belka C. Counting colonies of clonogenic assays by using densitometric software. Radiat Oncol. 2007; 2:4. https://doi.org/10.1186/1748-717X-2-4.

47. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics. 2003; 19:185–93. https://doi.org/10.1093/bioinformatics/19.2.185.

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REVIEW ARTICLE

Clipping the Wings of Glioblastoma: Modulation of WNT as a Novel Therapeutic Strategy

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Abstract

Glioblastoma (GBM) is the most malignant brain tumor and has a dismal prognosis. Aberrant WNT signaling is known to promote glioma cell growth and dissemination and resistance to conventional radio- and chemotherapy. Moreover, a population of cancer stem-like cells that promote glioma growth and recurrence are strongly dependent on WNT signaling. Here, we discuss the role and mechanisms of aberrant canonical and noncanonical WNT signaling in GBM. We present current clinical approaches aimed at modulating WNT activity and evaluate their clinical perspective as a novel treatment option for GBM.

Key Words: Glioma, Inhibitors, Noncanonical WNT signaling, Prognostic markers, Small molecules, WNT.

INTRODUCTION

Glioblastoma (GBM) is the most aggressive primary malignant brain tumor in adults. Despite continuous advancements in research, the median survival time of patients diagnosed with GBM remains less than 2 years (1). Current treatments combine operative resection of the tumor followed by radiation and chemotherapy with the alkylating agents temozolomide and/or carmustin (2, 3). For recurrent GBM, the vascular endothelial growth factor inhibitor bevacizumab is used, although recent studies have shown no significant benefit (4). The dismal prognosis is suggested to be a consequence of the existence of cancer stem-like cells (CSCs) that give rise to new glioma cells that can evolve and cause tumor recurrence, often with a more aggressive phenotype (5). This is at least partially due to the aberrant activation of developmentally conserved signaling pathways such as WNT (6–9). These pathways are also activated in neural stem cells during early development and in adulthood to promote their proliferation, self-renewal, and migration. Novel therapeutic strategies focus on selective targeting of CSC populations in gliomas by inhibiting these pathways (10, 11). Accumulating evidence suggests that the WNT signaling network acts as a potent oncogenic driver in some GBMs, and recent developments of effective, highly specific WNT inhibitors have raised hope for their clinical application as a therapeutic strategy in the near future.

Signal Transduction Along the WNT Pathway: Canonical and Noncanonical Signaling

WNT signaling can be divided into a canonical β-catenin-dependent branch and the noncanonical β-catenin-independent network (Fig. 1) . The canonical pathway is better understood. It is triggered by the binding of a WNT protein to the cell-surface receptor complex Frizzled (Fzd) and its coactivator, the lipoprotein receptor-related protein (LRP) 5/6. WNT binding enables the intracellular domain of Fzd complex with Dishevelled, which then acts as the key signaling mediator involved in the recruitment of Axin to the plasma membrane (Fig. 1). As a result of bound Axin, the β-catenin destruction complex (consisting of Axin, glycogen synthase kinase 3β [GSK-3β], and adenomatous-polyposis-coli [APC] protein), which is necessary to phosphorylate β-catenin, cannot assemble. Without WNT/Fzd interaction, the pathway is in "off-mode"; then, fully functional β-catenin-inactivation complex phosphorylates β-catenin, resulting in its ubiquitination and eventual degradation in the cells' proteasome. During WNT activation, β-catenin is stabilized and accumulates in the cytoplasm, where an oversaturation leads to its translocation into the nucleus. Intranuclear β-catenin interacts with transcription factors of the TCF/LEF family to promote target gene activation, thereby influencing a variety of cellular processes including proliferation, motility, and invasion (12).

The noncanonical WNT pathway is better known for its importance in body-axis formation and cell-fate specification, as well as migration during embryonic development (13, 14). Similarly, binding of WNT proteins to the Fzd receptors, allowing recruitment of Dishevelled, activates the noncanonical signaling. Subsequent release of calcium (Ca^{2+}) from the endoplasmic reticulum increases Nemo-like kinase (NLK), which in turn inhibits the β-catenin/TCF transcription complex. Ca^{2+} release also stimulates the transcription factor NFAT (nuclear factor of activated T cells), which enhances cell adhesion and migration (15). Ca^{2+}-independent noncanonical WNT signaling includes

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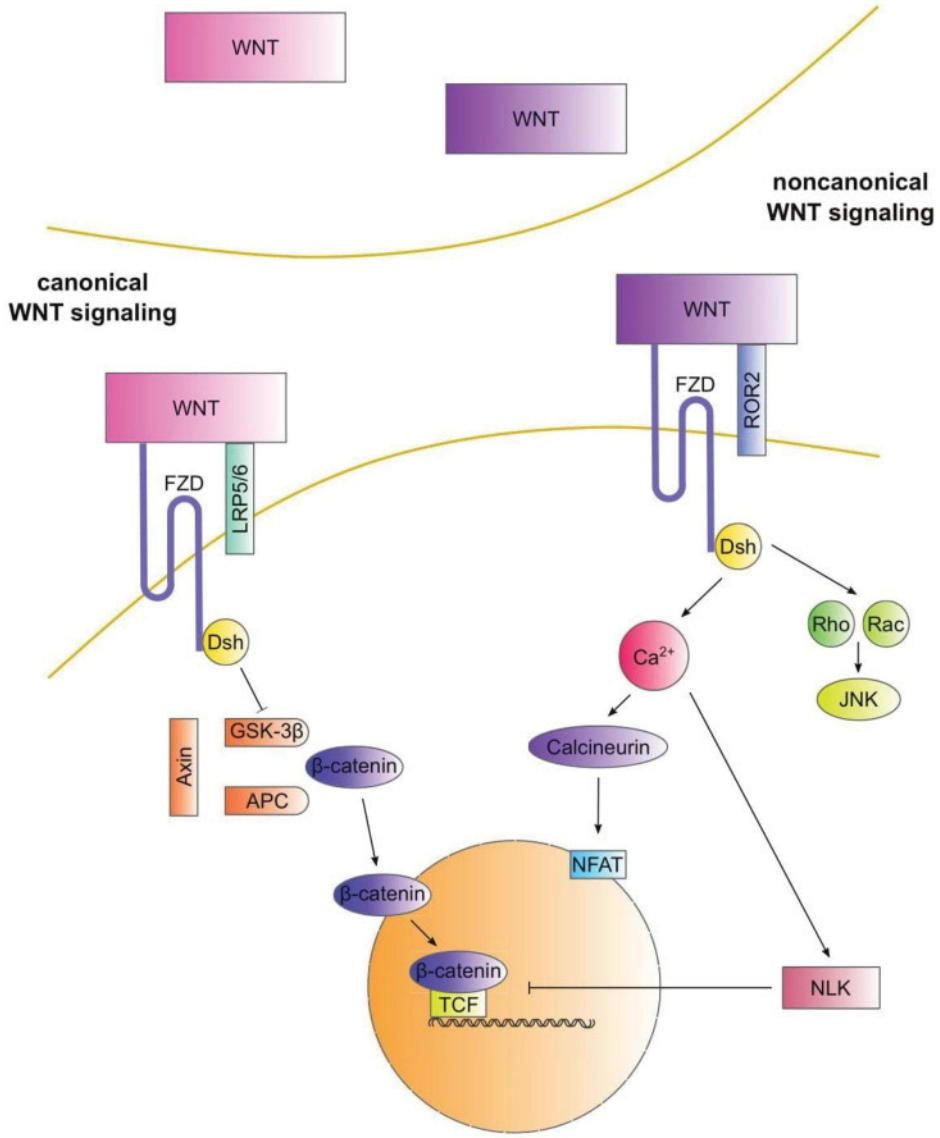


FIGURE 1. Canonical and noncanonical WNT signaling. The canonical WNT pathway is activated via binding of the WNT protein to the Frizzled and LRP5/6 receptors. Dishevelled inhibits β-catenin's destruction complex build of Axin, APC, and GSK-3β so that β-catenin accumulates in the cell and its nucleosome, where it drives target gene expression after binding to the LEF/TCF binding side. Activation of noncanonical WNT signaling is triggered through WNT proteins binding to the Frizzled receptor, whereas ROR2 acts as a coreceptor rather than LRP5/6. Activated through Frizzled, Dishevelled can either enhance Rho and Rac to activate the JNK pathway or increase the intracellular Ca^{2+} concentration. Increased Ca^{2+} enhances gene expression via NFAT and activates NLK that antagonizes β-catenin-mediated gene expression. WNTs-1, -2, -3, -8a, -8b, -10a, and -10b are supposed to activate the canonical/β-catenin pathway, whereas WNTs-4, -5a, -5b, -6, -7a, -7b, and 11 are defined to activate noncanonical signaling. FZD, Frizzled receptor; Dsh, Dishevelled; LRP5/6, lipoprotein-related protein; APC, adenomatous-polyposis-coli protein; GSK-3β, glycogen synthase kinase 3β; TCF, TCF/LEF binding side; Ca^{2+}, calcium; NFAT, nuclear factor of activated T-cells; NLK, Nemo-like kinase; Rho, Rho GTPase; Rac, ras-related C3 botulinum toxin substrate; JNK, c-Jun N-terminal protein kinase.

activation of the JNK (c-Jun N-terminal protein kinase), leading to planar cell polarity (16).

WNT Proteins

Until recently, it was not known which mechanisms determined which branch of the WNT network (ie, canonical or noncanonical) is activated. The initial hypothesis implied that the distinct WNT proteins activate only one side of the WNT pathway: WNT-1, -2, -3, -8a, -8b, -10a, and -10b are thought to activate the canonical pathway, whereas WNT-4, -5a, -5b, -6, -7a, -7b, and 11 are associated with the noncanonical cascade. WNTs-2b and -9b are undetermined (17, 18). However, WNT-3, which was traditionally considered canonical, was found to inhibit medulloblastoma cell proliferation via the noncanonical WNT pathway (19). Additionally, morphogens thought to belong to the noncanonical pathway (eg, WNT-7a) have been shown to stimulate canonical signaling (20); however, in GBMs, no such cross-over has been reported. Nevertheless, these observations indicate that the mechanisms governing the decision of whether canonical or noncanonical WNT signaling is activated are complex and do not depend solely on the WNT proteins interacting with their respective receptors.

WNT Receptors

Another concept suggests that the WNT receptors and their coreceptors determine whether the canonical or noncanonical pathway is activated (21). As mentioned before, secreted WNT proteins bind to a member of the 7-pass transmembrane receptor family called Frizzled, of which 10 different subtypes are described (21). To be effective, they have to bind additionally to the Fzd-coreceptor LRP5/6. This interaction is a classical activator of the canonical WNT pathway. Noncanonical signaling is activated if receptor-like tyrosine kinase (Ryk) and tyrosine-protein kinase transmembrane receptor (ROR2) are recruited as coreceptors. Thus, according to this model, not the secreted WNT proteins but rather the coreceptors to which they bind determine whether canonical or noncanonical signaling is activated. Nevertheless, some WNTs (eg, WNT-1 and -3a) have predilection to bind to LRP5/6 and some (eg, WNT-5a and -11) to Ryk or ROR2. The most recent study indicates that noncanonical WNTs (WNT-4 and -5a) are also able to induce canonical signaling, but solely with LRP5/6 overexpression. This study also demonstrated that WNT-4 and -5a are only capable of binding 3 out of 10 subtypes of Frizzled receptors (22). This might explain why WNT signaling has different influences on distinct cell types, suggesting that cells express an individual composition and different amounts of receptors. The overexpression of the Frizzled 4 receptor itself is related to the activation of the canonical WNT signaling, which increases stemness and invasiveness of GBM cell lines. In this context, it is not known whether Frizzled 4 has an effect on noncanonical WNT signaling (23). Moreover, Ryk, but not ROR2, leads to increased invasiveness in GBM that seems to be dependent on WNT-5a. In addition, Ryk and Fzd expression correlates with malignancy grade in GBM (24, 25).

Effect of Canonical and Noncanonical WNT Signaling in GBM

It is generally accepted that aberrant canonical WNT signaling leads to GBM progression. High β-catenin levels correlate with worse clinical outcome (26), and canonical WNT activation is described as a characteristic of CSCs in GBMs (27–29). Canonical WNT has been shown to mediate resistance to chemo- and radiotherapy (30–32). In epithelial cancers, WNT signaling acts as a major inducer of epithelial-to-mesenchymal transition (EMT). Studies suggest that, in gliomas classified as neuroepithelial tumors (33–37), a similar process can be observed leading to the so-called glial-mesenchymal transition (GMT) (38). GMT is related to migration and tumor spread by evoking single-cell movement (39, 40). The core-activator of EMT/GMT, *ZEB1*, is found to be a downstream target gene of canonical WNT signaling, representing a possible major mechanism by which WNT signaling augments glioma cell invasion (36, 41). However, another study claims that overexpression of β-catenin under hypoxia initiates neuronal differentiation of glioma CSCs. The authors explain this with a transcriptional drift of β-catenin cofactor expression under hypoxia, from *TCF4* toward *TCF1*/*LEF1*, a process known to occur in embryonic stem cells (42, 43). In that study, overexpression of β-catenin under hypoxia was linked to inhibition of Notch, which might explain suppression of stemness factors and induction of neuronal genes. The same effect has not been found relevant under normoxia (43). This finding, although preliminary, suggests that in vitro studies under normoxia might not reflect the physiological conditions of GBM, which are characterized by areas of severe hypoxia or anoxia.

Several recent studies have focused on the role of noncanonical WNT in different types of cancers (33, 44–46), but its importance in GBM is not yet well understood. Some reports show a negative correlation between canonical and noncanonical signaling, indicating a suppressive function of noncanonical signaling on β-catenin through activation of NLK (47–49). In GBM, cell migration appears to be regulated by noncanonical WNT signaling (25, 50–52). WNT-5a expression was also linked to increased proliferation in GBM as well as enhanced tumor formation capacity in xenografts (53). Both WNT-5a and -5b are frequently overexpressed in GBMs (25, 54).

Ligands that affect both the canonical and noncanonical WNT pathway, such as the WNT-inhibitor DKK3 or WNT-stimulator Evi, are reported to promote cellular proliferation (51, 55). Dishevelled 2, another protein interfering with both canonical and noncanonical signaling, causes differentiation and inhibits GBM growth in vivo. In that study, blocking of the canonical pathway reduced proliferation but did not promote differentiation (56). Taken together, our understanding of the effects of the noncanonical WNT pathway in malignant gliomas is still limited, and further investigations are needed to decipher its impact on the tumor cell biology.

Alterations of WNT Signaling in GBM

Aberrant WNT signaling is well known to play a crucial role in colon cancer and medulloblastoma, in which high WNT activity is caused by somatic mutations (57–60). A sub-

group of medulloblastomas is characterized by high WNT activation, mainly due to aberrant stabilization of β-catenin (61, 62). Interestingly, in contrast to GBM, high WNT is a positive clinical prognostic marker for medulloblastoma. In GBM, dysregulation of WNT signaling is mainly caused by deactivated pathway inhibitors (63). The WNT antagonist WIF-1 is silenced in more than one-third of all GBMs (64, 65). WIF-1 competitively binds WNT proteins, thus impeding their binding to the Frizzled/LRP receptor complex. When WIF-1 is expressed, it results in cell-cycle arrest in GBM cells (66). Consequently, one-third of all GBMs do not undergo cell-cycle arrest. Recent studies, however, indicate that WIF-1 does not only affect the canonical pathway, but also downregulates the noncanonical WNT/Ca^{2+} pathway (67). Dickkopf 1 (DKK1) and secreted Frizzled protein 1 (sFRP1), other canonical WNT-pathway antagonists, have been reported to be inactivated epigenetically in GBM; DKK1 also inhibits noncanonical signaling and was shown to reduce glioma-cell clonogenicity in vitro (68). The tumor suppressor gene *FAT1* inhibits glioma growth by binding β-catenin, thus preventing it from entering the nucleus. This recent finding extends the list of neoplasms in which WNT-activating somatic mutations can be found with a mutation frequency of about 1 out of 5 GBMs (69). Another gene that is silenced in GBM through promoter hypermethylation is *PEG3*. PEG3/Pw1 protein is involved in embryonic development, in which it leads to degradation of β-catenin (70). Interestingly, in GBM-CSCs, PEG3/Pw1 seems to be suppressed (71).

Aberrant autocrine-pathway regulation in GBM is best demonstrated through the WNT target gene *FOXM1*. The protein directly binds to β-catenin and promotes its translocation into the nucleus (72). In addition, *FOXM1* interacts with β-catenin and *STAT3* to promote GBM-CSC renewal, as shown in recent functional studies after genetic *FOXM1*-depletion in vitro. (73). Its role seems to be vital for glioma stem cell maintenance because inhibition through shRNA or small molecule inhibitors decreases multiple stem cell markers and sphere-forming capacity (74).

Based on data from the Cancer Genome Atlas, which subcategorizes GBMs according their different transcriptional signatures, the proneural subgroup is characterized by elevated expression of 2 WNT pathway activators, TCF4 and SOX. This group tends to have a bad prognosis and has higher incidences in younger patients (75–77). High expression of EMT activators and NF-κB, as well as canonical WNT targets *DKK1*, *Frizzled 1*, and *LEF1*, are described for the mesenchymal subgroup, which is also linked to very poor clinical outcome (36, 75). Taken together, these findings suggest that the WNT signaling network affects a variety of cellular processes and plays a fundamental role in gliomagenesis.

Prognostic Markers Related to WNT Signaling in GBM

Several studies report the overexpression of genes involved in the canonical WNT signaling pathway in gliomas, including *WNT1*, *β-catenin*, and *TCF4* (26, 77–80), suggesting their prognostic value. Analyzing the expression data from the Cancer Genome Atlas, *Axin2*, the most prominent WNT target gene, was recently reported to have a somewhat-negative prognostic value in GBM but did not reach statistical significance (78). In a histological approach, the amount of nuclear β-catenin, significantly higher in high-grade gliomas as compared to low-grade gliomas, correlates positively with WHO grade (26, 81). The expression level of the noncanonical WNT ligand WNT-4 correlates negatively with WHO grade (82). At present, none of these markers are used in routine clinical practice. Should pharmaceutical WNT inhibition be considered as a relevant therapy option for gliomas, inclusion of direct WNT pathway members or downstream mediators for diagnostic purposes should be evaluated carefully through more comprehensive retrospective and prospective studies.

Pharmacological WNT Modulation

Given the significant accumulation of strong preclinical data documenting the importance of WNT signaling in tumor biology, the pharmaceutical industry has defined the development of potent and specific WNT inhibitors as a main goal for future therapy in oncology. Many of the new compounds have already been tested successfully in cancers with known WNT mutations (83–85). In this section, we focus our discussion on small-molecule inhibitors and pharmacological compounds that have been tested in GBM in vitro and partly in vivo.

A small-molecule porcupine inhibitor, LGK974, blocks the indispensable palmitoylation of WNT proteins, thus preventing their secretion and binding to the receptor complex (Table). This substance has effectively inhibited WNT and reduced tumor growth in preclinical models of pancreatic and head and neck cancers (86, 87). Recent studies indicate that LGK974 inhibits canonical WNT in glioma cells and results in decreased proliferation, survival as well as a depletion of the CSC pool in vitro (78). Due to the fact that LGK974 modulates the secretion of all WNT proteins, inhibition of both canonical and noncanonical pathways is likely.

Another compound, SEN461, effectively reduces glioma growth in vitro and in vivo, possibly through the stabilization of Axin, thus augmenting the degradation of β-catenin (88, 89); however, its precise site and mechanism of action of is currently still unknown.

Two other compounds currently in preclinical development, XAV939 and IWR2, stabilize Axin through the inhibition of tankyrase, an enzyme that stimulates Axin degradation in the proteasome (90–92). Both molecules show antitumorigenic effects in vitro but have yet to be tested in a clinical trial (93).

Another therapeutic target influencing the β-catenin destruction complex is Aurora-A, a seronine/threonine kinase that interacts with Axin and destabilizes the GSK3β/Axin/β-catenin structure. Originally, Aurora-A was found to be localized at centrosomes of cancer cells, promoting their mitosis (94). Current phase III studies are investigating the Aurora-A-inhibitor alisertib in patients with T-cell lymphoma, but results, though promising, are not yet conclusive. Also, in GBM, alisertib was found to decrease WNT signaling in vitro and in vivo, and aurora-A-knockdown targeted the GBM-CSC compartment (95–97).

TABLE. Inhibitors and Activators of WNT Signaling in Glioblastomas

WNT Inhibitors	Target	Mechanism	Development Stage in Cancer	Research Stage in GBM	Reference
LGK974	Porcupine	Inhibition of palmitoylation of WNT proteins	Phase I	In vitro	(78, 86, 87)
SEN461	Axin	Axin stabilization through tankyrase inhibition	Preclinical	In vivo	(88, 89, 92)
XAV939	Tankyrase	Axin stabilization through tankyrase inhibition	Preclinical	In vitro	(90–93)
IWR2	Tankyrase	Axin stabilization through tankyrase inhibition	Preclinical	In vitro	(90–93)
Aurora-A-inhibitor	Axin	Stabilization of destruction complex	Phase III	In vivo	(95–97)
ICG-001	CBP	Disruption of CBP/β-catenin complex	Preclinical	In vitro	(83, 100)
PKF115-584	β-catenin/TCF	Disruption of transcription complex	Preclinical	In vitro	(99, 100)
Aspirin	COX	Repression of gene transcription	–	In vitro	(101, 102)
FH535	β-catenin/TCF/LEF	Disruption of transcription complex	Preclinical	In vivo	(104, 105)
BASI	miR-200a, miR-181d	Inactivation of β-catenin	Preclinical	In vitro	(112)
Enzastaurin	PKCβ	Inhibition of GSK-3β	Phase III	In vivo	(107–111)
Lithium chloride	GSK-3β	Inhibition of GSK-3β	Preclinical	In vitro	(113)

CBP, CREB-binding protein; GSK-3β, glycogen synthase kinase 3β; LEF, lymphoid enhancer factor; miR, microRNA; PKCβ, protein kinase C β; TCF, T-cell factor.

Many new drugs aim to target specifically the β-catenin-transcription-complex. ICG-001, for example, targets the NH_2-terminus of *CBP*, thus disturbing its interaction with β-catenin and therefore impeding an effective downstream signal transduction (98). PKF115-584 also specifically interrupts the β-catenin-TCF complex (99). Both compounds induced differentiation and reduced cellular proliferation in GBM cultures (100). A second-generation CBP-inhibitor PRI-724 is very effective in low concentrations but has not yet been tested on glioma cells.

Interestingly, the prominent analgesic and antiphlogistic drug aspirin was demonstrated to suppress the effect of WNT signaling. Similar to an NSAID and an irreversible COX inhibitor, aspirin reduces inflammatory mediators and diminishes WNT pathway activity in colon cancer by attenuating the transcription of β-catenin/TCF-responsive genes (101, 102). The same effect was observed in glioma cell lines (103). However, because of its broad effects on various other biological processes and high molecular concentration needed to suppress WNT, further clinical testing as a treatment option for patients with GBM is needed.

FH535 is a dual inhibitor that targets both peroxisome proliferator-activated receptor (PPAR) and the β-catenin/TCF/LEF complex. Its inhibitory effect on canonical WNT signaling is assumed to be the result of repressing the recruitment of β-catenin and coactivator glutamate receptor interacting protein 1 (GRIP1) (104). Promisingly, FH535 downregulates β-catenin and TCF in glioma cell both in vitro and in vivo (105). It is unknown why these results did not lead to clinical tests, but the authors speculate that the limited knowledge of the mechanism of action of FH535 might be the reason.

The compound Enzastaurin, an inhibitor of GSK-3β, is currently in a phase III clinical trial for patients with B-cell lymphoma. As an inhibitor of protein kinase Cβ (PKCβ), Enzastaurin prevents phosphorylation of GSK-3β and AKT. Inactivation of these proteins in tumor cells reduces proliferation and impacts tumor angiogenesis. This effect has also been observed in GBM xenografts (106, 107). A phase III study testing Enzastaurin versus the nitrosourea-derived alkylating agent Lomustine on patients with GBM failed to prove any benefit; however, the therapy was well tolerated (108). Enzastaurin was additionally tested in combination with temozolomide and irradiation on the basis of preclinical studies indicating synergistic effects (109, 110). Nevertheless, Enzastaurin has not yet made it into clinical application (111–113).

Clinical Perspective of WNT-Targeted Therapies in Gliomas

Over the last several years, the role of WNT in gliomagenesis has become increasingly appreciated (114). In this review, we discuss the current understanding of canonical and noncanonical WNT signaling in gliomas. Despite numerous studies indicating the activation of WNT signaling as an important piece of gliomagenesis, the "big picture" is far from clear. For example, canonical WNT signaling in combination with hypoxia was described to reduce stemness in GBM. These data could be supported by other studies in stem and progenitor cells, indicating that canonical WNT signaling can act as a driver of stem cell differentiation (43, 115–117). Since most studies describe WNT pathway activation to promote stem cells in cancer, these results appear controversial and require

further investigation of the underlying molecular events following WNT activation. Regarding GBM, tumor environment seems to play a pivotal role and might impact WNT signaling; therefore, we need to study the biology of WNT inhibition under hypoxia. Investigation of WNT inhibition only under normoxia might be missing potential side effects.

In addition, the WNT pathway participates in active reciprocal dialogue with other important cell-signaling cascades, including AKT, Hippo, Notch, Hedgehog, and epithelial growth factor receptor (EGFR) (118–121). Accordingly, modulating one of these pathways may influence WNT, and WNT modulation could affect other pathways, resulting in the development of treatment resistance. To better evaluate the impact of new WNT pathway inhibitors, the potential side effects need to be carefully investigated.

We describe WNT-associated molecules serving as potential prognostic biomarkers for GBM. These findings provide some support for the conceptual premise that GBM malignancy correlates with high canonical WNT activation but still need to be interpreted with caution. With confirmation in larger clinical datasets, these markers could be used to monitor anti-WNT therapy. Because not all GBMs show high aberrant WNT activation, further work is required to find reliable predictor markers to define GBMs for which blockade of WNT might be a suitable therapeutic option.

A major physiological problem in reaching glioma cells remains the blood-brain barrier (BBB), which is a mechanical obstacle for the majority of drugs. This should affect the design of molecules with specific physiochemical properties, including high lipophilicity, small size, and a long half-life period, to increase the likelihood of penetrating the BBB (122). In addition, osmotic BBB disruption or combination of the active substances with nanoparticles may prove successful in treating glioma patients with drugs otherwise incapable of entering the brain. The recently described application of focused ultrasound technology that temporarily and locally opens the BBB is another promising strategy to enhance intra-CNS drug delivery (123).

At this point in time, WNT inhibitors are being tested in clinical trials in WNT-dependent cancers, and none of them have been successfully tested in glioma patients. It is conceivable that WNT inhibitors may be used in combination with temozolomide and irradiation because WNT signaling is linked to glioma resistance against conventional chemo- and radiotherapy.

As shown, most developed drugs aim to target only the canonical WNT pathway. In this review, we report that both WNT pathways (ie, the canonical and the noncanonical) correlate with GBM malignancy and disease progression. These findings are consistent with reports in other cancer types (124–127). In colon cancer, cells even undergo a switch from canonical to noncanonical WNT signaling to enhance drug resistance (128). According to these data, we can infer that combating GBM might be more effective by targeting both canonical and noncanonical WNT signaling simultaneously using single- or multiagent therapeutic approaches. Among the pharmaceuticals discussed herein, only LGK974 fulfills this qualification. To develop a full picture of LGK974's potential in treating GBM, additional studies will be needed that evaluate its permeability through the BBB and effectiveness in vivo.

REFERENCES

1. Sacko A, Hou M-M, Temgoua M, et al. Evolution of the Karnosky performance status throughout life in glioblastoma patients. J Neurooncol 2015;122:5671
2. Nava F, Tramacere I, Fittipaldo A, et al. Survival effect of first- and second-line treatments for patients with primary glioblastoma: A cohort study from a prospective registry, 1997-2010. Neuro Oncol 2014;16: 719–27
3. Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005;352:987–96
4. Hamza MA, Mandel JJ, Conrad CA, et al. Survival outcome of early versus delayed bevacizumab treatment in patients with recurrent glioblastoma. J Neurooncol 2014;119:135–40
5. Schonberg DL, Lubelski D, Miller TE, et al. Brain tumor stem cells: Molecular characteristics and their impact on therapy. Mol Aspects Med 2014;39:82–101
6. Rheinbay E, Suvà ML, Gillespie SM, et al. An aberrant transcription factor network essential for Wnt signaling and stem cell maintenance in glioblastoma. Cell Rep 2013;3:1567–79
7. Saito N, Aoki K, Hirai N, et al. Effect of Notch expression in glioma stem cells on therapeutic response to chemo-radiotherapy in recurrent glioblastoma. Brain Tumor Pathol 2015;32:176–83
8. Takezaki T, Hide T, Takanaga H, et al. Essential role of the Hedgehog signaling pathway in human glioma-initiating cells. Cancer Sci 2011;102: 1306–12
9. Qiang L, Yang Y, Ma Y-J, et al. Isolation and characterization of cancer stem like cells in human glioblastoma cell lines. Cancer Lett 2009;279: 13–21
10. Dai L, Liu Y, He J, et al. Differential profiling studies of N-linked glycoproteins in glioblastoma cancer stem cells upon treatment with γ-secretase inhibitor. Proteomics 2011;11:4021–8
11. Ding D, Lim KS, Eberhart CG. Arsenic trioxide inhibits Hedgehog, Notch and stem cell properties in glioblastoma neurospheres. Acta Neuropathol Commun 2014;2:31
12. Clevers H, Loh KM, Nusse R. Stem cell signaling. An integral program for tissue renewal and regeneration: Wnt signaling and stem cell control. Science 2014;346:1248012
13. Kühl M, Sheldahl LC, Park M, et al. The Wnt/Ca2 + pathway: A new vertebrate Wnt signaling pathway takes shape. Trends Genet 2000;16: 279–83
14. Nüsslein-Volhard C. Determination of the embryonic axes of Drosophila. Dev Camb Engl Suppl 1991;1:1–10
15. Jauliac S, López-Rodriguez C, Shaw LM, et al. The role of NFAT transcription factors in integrin-mediated carcinoma invasion. Nat Cell Biol 2002;4:540–4
16. Grumolato L, Liu G, Mong P, et al. Canonical and noncanonical Wnts use a common mechanism to activate completely unrelated coreceptors. Genes Dev 2010;24:2517–30
17. Liu F, Millar SE. Wnt/beta-catenin signaling in oral tissue development and disease. J Dent Res 2010;89:318–30
18. Siar CH, Nagatsuka H, Han PP, et al. Differential expression of canonical and non-canonical Wnt ligands in ameloblastoma. J Oral Pathol Med 2012;41:332–9
19. Anne SL, Govek E-E, Ayrault O, et al. WNT3 inhibits cerebellar granule neuron progenitor proliferation and medulloblastoma formation via MAPK activation. PLoS One 2013;8:e81769
20. Yoshioka S, King ML, Ran S, et al. WNT7A regulates tumor growth and progression in ovarian cancer through the WNT/β-catenin pathway. Mol Cancer Res 2012;10:469–82
21. Van Amerongen R, Mikels A, Nusse R. Alternative WNT signaling is initiated by distinct receptors. Sci Signal 2008;1:re9
22. Ring L, Neth P, Weber C, et al. β-Catenin-dependent pathway activation by both promiscuous "canonical" WNT3a-, and specific "noncanonical" WNT4- and WNT5a-FZD receptor combinations with strong differences in LRP5 and LRP6 dependency. Cell Signal 2014;26:260–7
23. Jin X, Jeon H-Y, Joo KM, et al. Frizzled 4 regulates stemness and invasiveness of migrating glioma cells established by serial intracranial transplantation. Cancer Res 2011;71:3066–75

24. Habu M, Koyama H, Kishida M, et al. Ryk is essential for Wnt-5a-dependent invasiveness in human glioma. J Biochem 2014;156:29–38
25. Kamino M, Kishida M, Kibe T, et al. Wnt-5a signaling is correlated with infiltrative activity in human glioma by inducing cellular migration and MMP-2. Cancer Sci 2011;102:540–8
26. Schüle R, Dictus C, Campos B, et al. Potential canonical wnt pathway activation in high-grade astrocytomas. Sci World J 2012;2012: 697313
27. Sandberg CJ, Altschuler G, Jeong J, et al. Comparison of glioma stem cells to neural stem cells from the adult human brain identifies dysregulated Wnt- signaling and a fingerprint associated with clinical outcome. Exp Cell Res 2013;319:2230–43
28. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. Nature 2004;432:396–401
29. Kaur N, Chettiar S, Rathod S, et al. Wnt3a mediated activation of Wnt/β-catenin signaling promotes tumor progression in glioblastoma. Mol Cell Neurosci 2013;54:44–57
30. Hu G, Wei Y, Kang Y. The multifaceted role of MTDH/AEG-1 in cancer progression. Clin Cancer Res 2009;15:5615–20
31. Kim Y, Kim KH, Lee J, et al. Wnt activation is implicated in glioblastoma radioresistance. Lab Invest 2012;92:466–73
32. Riganti C, Salaroglio IC, Caldera V, et al. Temozolomide downregulates P-glycoprotein expression in glioblastoma stem cells by interfering with the Wnt3a/glycogen synthase-3 kinase/β-catenin pathway. Neuro Oncol 2013;15:1502–17
33. Bo H, Zhang S, Gao L, et al. Upregulation of Wnt5a promotes epithelial-to-mesenchymal transition and metastasis of pancreatic cancer cells. BMC Cancer 2013;13:496
34. Boyer B, Vallés AM, Edme N. Induction and regulation of epithelial-mesenchymal transitions. Biochem Pharmacol 2000;60:1091–9
35. Hotz B, Arndt M, Dullat S, et al. Epithelial to mesenchymal transition: Expression of the regulators snail, slug, and twist in pancreatic cancer. Clin Cancer Res 2007;13:4769–76
36. Kahlert UD, Maciaczyk D, Doostkam S, et al. Activation of canonical WNT/β-catenin signaling enhances in vitro motility of glioblastoma cells by activation of ZEB1 and other activators of epithelial-to-mesenchymal transition. Cancer Lett 2012;325:42–53
37. Wang Z, Zhang S, Siu TL, et al. Glioblastoma multiforme formation and EMT: Role of FoxM1 transcription factor. Curr Pharm Des 2015; 21:1268–71
38. Mahabir R, Tanino M, Elmansuri A, et al. Sustained elevation of Snail promotes glial-mesenchymal transition after irradiation in malignant glioma. Neuro Oncol 2014;16:671–85
39. Birchmeier C, Birchmeier W, Brand-Saberi B. Epithelial-mesenchymal transitions in cancer progression. Acta Anat 1996;156:217–26
40. Hay ED. An overview of epithelio-mesenchymal transformation. Acta Anat 1995;154:8–20
41. Kahlert UD, Suwala AK, Raabe EH, et al. ZEB1 promotes invasion in human fetal neural stem cells and hypoxic glioma neurospheres. Brain Pathol 2015;25:724–32
42. Mazumdar J, O'Brien WT, Johnson RS, et al. O_2 regulates stem cells through Wnt/β-catenin signalling. Nat Cell Biol 2010;12:1007–13
43. Rampazzo E, Persano L, Pistollato F, et al. Wnt activation promotes neuronal differentiation of glioblastoma. Cell Death Dis 2013;4:e500
44. Dejmek J, Dejmek A, Säfholm A, et al. Wnt-5a protein expression in primary dukes B colon cancers identifies a subgroup of patients with good prognosis. Cancer Res 2005;65:9142–6
45. Kremenevskaja N, von Wasielewski R, Rao AS, et al. Wnt-5a has tumor suppressor activity in thyroid carcinoma. Oncogene 2005;1: 2144–54
46. Kurayoshi M, Oue N, Yamamoto H, et al. Expression of Wnt-5a is correlated with aggressiveness of gastric cancer by stimulating cell migration and invasion. Cancer Res 2006;66:10439–48
47. Florian MC, Nattamai KJ, Dörr K, et al. A canonical to non-canonical Wnt signalling switch in haematopoietic stem-cell ageing. Nature 2013; 503:392–6
48. Topol L, Jiang X, Choi H, et al. Wnt-5a inhibits the canonical Wnt pathway by promoting GSK-3-independent beta-catenin degradation. J Cell Biol 2003;162:899–908
49. Yuan Y, Niu CC, Deng G, et al. The Wnt5a/Ror2 noncanonical signaling pathway inhibits canonical Wnt signaling in K562 cells. Int J Mol Med 2011;27:63–9
50. Cherry EM, Lee DW, Jung J-U, et al. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) promotes glioma cell invasion through induction of NF-κB-inducing kinase (NIK) and noncanonical NF-κB signaling. Mol Cancer 2015;14:9
51. Augustin I, Goidts V, Bongers A, et al. The Wnt secretion protein Evi/Gpr177 promotes glioma tumourigenesis. EMBO Mol Med 2012;4: 38–51
52. Tchoghandjian A, Jennewein C, Eckhardt I, et al. Identification of non-canonical NF-κB signaling as a critical mediator of Smac mimetic-stimulated migration and invasion of glioblastoma cells. Cell Death Dis 2013;4:e564
53. Yu JM, Jun ES, Jung JS, et al. Role of Wnt5a in the proliferation of human glioblastoma cells. Cancer Lett 2007;257:172–81
54. Schiffman JD, Hodgson JG, VandenBerg SR, et al. Oncogenic BRAF mutation with CDKN2A inactivation is characteristic of a subset of pediatric malignant astrocytomas. Cancer Res 2010;70:512–9
55. Hara K, Kageji T, Mizobuchi Y, et al. Blocking of the interaction between Wnt proteins and their co-receptors contributes to the anti-tumor effects of adenovirus-mediated DKK3 in glioblastoma. Cancer Lett 2015;356:496–505
56. Pulvirenti T, Van Der Heijden M, Droms LA, et al. Dishevelled 2 signaling promotes self-renewal and tumorigenicity in human gliomas. Cancer Res 2011;71:7280–90
57. Dahmen RP, Koch A, Denkhaus D, et al. Deletions of AXIN1, a component of the WNT/wingless pathway, in sporadic medulloblastomas. Cancer Res 2001;61:7039–43
58. Eberhart CG, Tihan T, Burger PC. Nuclear localization and mutation of beta-catenin in medulloblastomas. J Neuropathol Exp Neurol 2000;59: 333–7
59. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990;61:759–67
60. Huang H, Mahler-Araujo BM, Sankila A, et al. APC mutations in sporadic medulloblastomas. Am J Pathol 2000;156:433–7
61. Ellison DW, Onilude OE, Lindsey JC, et al. Beta-catenin status predicts a favorable outcome in childhood medulloblastoma: The United Kingdom Children's Cancer Study Group Brain Tumour Committee. J Clin Oncol 2005;23:7951–7
62. Gajjar A, Chintagumpala M, Ashley D, et al. Risk-adapted craniospinal radiotherapy followed by high-dose chemotherapy and stem-cell rescue in children with newly diagnosed medulloblastoma (St Jude Medulloblastoma-96): Long-term results from a prospective, multicentre trial. Lancet Oncol 2006;7:813–20
63. Götze S, Wolter M, Reifenberger G, et al. Frequent promoter hypermethylation of Wnt pathway inhibitor genes in malignant astrocytic gliomas. Int J Cancer 2010;126:2584–93
64. Yang Z, Wang Y, Fang J, et al. Downregulation of WIF-1 by hypermethylation in astrocytomas. Acta Biochim Biophys Sin 2010;42:418–25
65. Lambiv WL, Vassallo I, Delorenzi M, et al. The Wnt inhibitory factor 1 (WIF1) is targeted in glioblastoma and has a tumor suppressing function potentially by induction of senescence. Neuro Oncol 2011;131: 47
66. Wu J, Fang J, Yang Z, et al. Wnt inhibitory factor-1 regulates glioblastoma cell cycle and proliferation. J Clin Neurosci 2012;19:1428–32
67. Vassallo I, Zinn P, Lai M, et al. WIF1 re-expression in glioblastoma inhibits migration through attenuation of non-canonical WNT signaling by downregulating the lncRNA MALAT1. Oncogene 2016;35:12–21
68. Foltz G, Yoon J-G, Lee H, et al. Epigenetic regulation of wnt pathway antagonists in human glioblastoma multiforme. Genes Cancer 2010;1: 81–90
69. Morris LGT, Kaufman AM, Gong Y, et al. Recurrent somatic mutation of FAT1 in multiple human cancers leads to aberrant Wnt activation. Nat Genet 2013;45:253–61
70. El Kharroubi A, Piras G, Stewart CL. DNA demethylation reactivates a subset of imprinted genes in uniparental mouse embryonic fibroblasts. J Biol Chem 2001;276:8674–80
71. Jiang X, Yu Y, Yang HW, et al. The imprinted gene PEG3 inhibits Wnt signaling and regulates glioma growth. J Biol Chem 2010;285:8472–80
72. Zhang N, Wei P, Gong A, et al. FoxM1 promotes β-catenin nuclear localization and controls Wnt target-gene expression and glioma tumorigenesis. Cancer Cell 2011;20:427–42
73. Gong A-H, Wei P, Zhang S, et al. FoxM1 drives a feed-forward STAT3 activation loop that promotes self-renewal and tumorigenicity of glioblastoma stem-like cells. Cancer Res 2015;75:2337–48

74. Nitta RT, Gholamin S, Feroze AH, et al. Casein kinase 2α regulates glioblastoma brain tumor-initiating cell growth through the β-catenin pathway. Oncogene 2015;34:3688–99
75. Verhaak RGW, Hoadley KA, Purdom E, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. Cancer Cell 2010;17:98–110
76. Phillips HS, Kharbanda S, Chen R, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. Cancer Cell 9:157–73
77. Chen L, Huang K, Han L, et al. β-catenin/TCF-4 complex transcriptionally regulates AKT1 in glioma. Int J Oncol 2011;39:883–90
78. Kahlert UD, Suwala AK, Koch K, et al. Pharmacologic Wnt inhibition reduces proliferation, survival, and clonogenicity of glioblastoma cells. J Neuropathol Exp Neurol 2015;74:889–900
79. Liu C, Tu Y, Sun X, et al. Wnt/beta-Catenin pathway in human glioma: Expression pattern and clinical/prognostic correlations. Clin Exp Med 2011;11:105–12
80. Rossi M, Magnoni L, Miracco C, et al. β-catenin and Gli1 are prognostic markers in glioblastoma. Cancer Biol Ther 2011;11:753–61
81. Wu W, Tian Y, Wan H, et al. The expressions of Wnt/β-catenin pathway-related components in brainstem gliomas. Can J Neurol Sci 2013; 40:355–60
82. Shen F, Zhang Y, Yao Y, et al. Proteomic analysis of cerebrospinal fluid: Toward the identification of biomarkers for gliomas. Neurosurg Rev 2014;37:367–80
83. Grigson ER, Ozerova M, Pisklakova A, et al. Canonical Wnt pathway inhibitor ICG-001 induces cytotoxicity of multiple myeloma cells in Wnt-independent manner. PLoS One 2015;10:e0117693
84. Jang G-B, Hong I-S, Kim R-J, et al. Wnt/β-catenin small molecule inhibitor CWP232228 preferentially inhibits the growth of breast cancer stem-like cells. Cancer Res 2015;75:1691–702
85. Mallinger A, Crumpler S, Pichowicz M, et al. Discovery of potent, orally bioavailable, small-molecule inhibitors of WNT signaling from a cell-based pathway screen. J Med Chem 2015;58:1717–35
86. Jiang X, Hao H-X, Growney JD, et al. Inactivating mutations of RNF43 confer Wnt dependency in pancreatic ductal adenocarcinoma. Proc Natl Acad Sci U S A 2013;110:12649–54
87. Liu J, Pan S, Hsieh MH, et al. Targeting Wnt-driven cancer through the inhibition of Porcupine by LGK974. Proc Natl Acad Sci U S A 2013; 110:20224–9
88. De Robertis A, Mennillo F, Rossi M, et al. Human sarcoma growth is sensitive to small-molecule mediated AXIN stabilization. PLoS One 2014;9:e97847
89. Nencini A, Pratelli C, Quinn JM, et al. Structure-activity relationship and properties optimization of a series of quinazoline-2, 4-diones as inhibitors of the canonical Wnt pathway. Eur J Med Chem 2015;95:526–45
90. Chen B, Dodge ME, Tang W, et al. Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. Nat Chem Biol 2009;5:100–7
91. Huang S-MA, Mishina YM, Liu S, et al. Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. Nature 2009;461:614–20
92. Gunaydin H, Gu Y, Huang X. Novel binding mode of a potent and selective tankyrase inhibitor. PLoS One 2012;7:e33740
93. De Robertis A, Valensin S, Rossi M, et al. Identification and characterization of a small-molecule inhibitor of Wnt signaling in glioblastoma cells. Mol Cancer Ther 2013;12:1180–9
94. Friedberg JW, Mahadevan D, Cebula E, et al. Phase II study of alisertib, a selective Aurora A kinase inhibitor, in relapsed and refractory aggressive B- and T-cell non-Hodgkin lymphomas. J Clin Oncol 2014;32:44–50
95. Lee P-Y, Chen C-L, Lin Z-Z, et al. The Aurora kinases inhibitor VE-465 is a novel treatment for glioblastoma multiforme. Oncology 2013; 84:326–35
96. Van Brocklyn JR, Wojton J, Meisen WH, et al. Aurora-A inhibition offers a novel therapy effective against intracranial glioblastoma. Cancer Res 2014;74:5364–70
97. Xia Z, Wei P, Zhang H, et al. AURKA governs self-renewal capacity in glioma-initiating cells via stabilization/activation of β-catenin/Wnt signaling. Mol Cancer Res 2013;11:1101–11
98. Emami KH, Nguyen C, Ma H, et al. A small molecule inhibitor of beta-catenin/CREB-binding protein transcription [corrected]. Proc Natl Acad Sci U S A 2004;101:12682–7
99. Sukhdeo K, Mani M, Zhang Y, et al. Targeting the beta-catenin/TCF transcriptional complex in the treatment of multiple myeloma. Proc Natl Acad Sci U S A 2007;104:7516–21
100. Huang SC, Clarke DC, Gosline SJC, et al. Linking proteomic and transcriptional data through the interactome and epigenome reveals a map of oncogene-induced signaling. PLoS Comput Biol 2013;9:e1002887
101. Dihlmann S, Siermann A, von Knebel Doeberitz M. The nonsteroidal anti-inflammatory drugs aspirin and indomethacin attenuate beta-catenin/TCF-4 signaling. Oncogene 2001;20:645–53
102. Dihlmann S, Klein S, Doeberitz Mv Mv. Reduction of beta-catenin/T-cell transcription factor signaling by aspirin and indomethacin is caused by an increased stabilization of phosphorylated beta-catenin. Mol Cancer Ther 2003;2:509–16
103. Lan F, Yue X, Han L, et al. Antitumor effect of aspirin in glioblastoma cells by modulation of β-catenin/T-cell factor-mediated transcriptional activity. J Neurosurg 2011;115:780–8
104. Handeli S, Simon JA. A small-molecule inhibitor of TCF/beta-catenin signaling down-regulates PPARgamma and PPARdelta activities. Mol Cancer Ther 2008;7:521–9
105. Shi Z, Qian X, Li L, et al. Nuclear translocation of β-catenin is essential for glioma cell survival. J Neuroimmune Pharmacol 2012;7:892–903
106. Graff JR, McNulty AM, Hanna KR, et al. The protein kinase Cbeta-selective inhibitor, Enzastaurin (LY317615.HCl), suppresses signaling through the AKT pathway, induces apoptosis, and suppresses growth of human colon cancer and glioblastoma xenografts. Cancer Res 2005;65: 7462–9
107. Kreisl TN, Kim L, Moore K, et al. A phase I trial of enzastaurin in patients with recurrent gliomas. Clin Cancer Res 2009;15:3617–23
108. Wick W, Puduvalli VK, Chamberlain MC, et al. Phase III study of enzastaurin compared with lomustine in the treatment of recurrent intracranial glioblastoma. J Clin Oncol 2010;28:1168–74
109. Butowski N, Chang SM, Lamborn KR, et al. Phase II and pharmacogenomics study of enzastaurin plus temozolomide during and following radiation therapy in patients with newly diagnosed glioblastoma multiforme and gliosarcoma. Neuro Oncol 2011;13:1331–8
110. Tabatabai G, Frank B, Wick A, et al. Synergistic antiglioma activity of radiotherapy and enzastaurin. Ann Neurol 2007;61:153–61
111. Shi Z-D, Qian X-M, Zhang J-X, et al. BASI, a potent small molecular inhibitor, inhibits glioblastoma progression by targeting microRNA-mediated β-catenin signaling. CNS Neurosci Ther 2014;20:830–9
112. Atkins RJ, Dimou J, Paradiso L, et al. Regulation of glycogen synthase kinase-3 beta (GSK-3β) by the Akt pathway in gliomas. J Clin Neurosci 2012;19:1558–63
113. Chou C-H, Chou A-K, Lin C-C, et al. GSK3β regulates Bcl2L12 and Bcl2L12A anti-apoptosis signaling in glioblastoma and is inhibited by LiCl. Cell Cycle 2012;11:532–42
114. Anastas JN, Moon RT. WNT signalling pathways as therapeutic targets in cancer. Nat Rev Cancer 2013;13:11–26
115. Kim J-A, Choi H-K, Kim T-M, et al. Regulation of mesenchymal stromal cells through fine tuning of canonical Wnt signaling. Stem Cell Res 2015;14:356–68
116. Tamura M, Uyama M, Sugiyama Y, et al. Canonical Wnt signaling activates miR-34 expression during osteoblastic differentiation. Mol Med Rep 2013;8:1807–11
117. Wang L, Liu Y, Li S, et al. Wnt signaling pathway participates in valproic acid-induced neuronal differentiation of neural stem cells. Int J Clin Exp Pathol 2015;8:578–85
118. Tzeng H-E, Yang L, Chen K, et al. The pan-PI3K inhibitor GDC-0941 activates canonical WNT signaling to confer resistance in TNBC cells: Resistance reversal with WNT inhibitor. Oncotarget 2015;6:11061–73
119. Shimobayashi M, Hall MN. Making new contacts: The mTOR network in metabolism and signalling crosstalk. Nat Rev Mol Cell Biol 2014;15: 155–62
120. Collu GM, Hidalgo-Sastre A, Brennan K. Wnt-Notch signalling crosstalk in development and disease. Cell Mol Life Sci 2014;71:3553–67
121. Paul I, Bhattacharya S, Chatterjee A, et al. Current understanding on EGFR and Wnt/β-catenin signaling in glioma and their possible crosstalk. Genes Cancer 2013;4:427–46
122. Seelig A, Gottschlich R, Devant RM. A method to determine the ability of drugs to diffuse through the blood-brain barrier. Proc Natl Acad Sci U S A 1994;91:68–72

123. Liu H-L, Hua M-Y, Chen P-Y, et al. Blood-brain barrier disruption with focused ultrasound enhances delivery of chemotherapeutic drugs for glioblastoma treatment. Radiology 2010;255:415–25
124. Lu C, Wang X, Zhu H, et al. Over-expression of ROR2 and Wnt5a cooperatively correlates with unfavorable prognosis in patients with non-small cell lung cancer. Oncotarget 2015;6:24912–21
125. Janovska P, Poppova L, Plevova K, et al. Autocrine signaling by Wnt-5a deregulates chemotaxis of leukemic cells and predicts clinical outcome in chronic lymphocytic leukemia. Clin Cancer Res 2016;22: 459–69
126. Qin L, Yin Y-T, Zheng F-J, et al. WNT5A promotes stemness characteristics in nasopharyngeal carcinoma cells leading to metastasis and tumorigenesis. Oncotarget 2015;6:10239–52
127. Lin L, Liu Y, Zhao W, et al. Wnt5A expression is associated with the tumor metastasis and clinical survival in cervical cancer. Int J Clin Exp Pathol 2014;7:6072–8
128. Bordonaro M, Tewari S, Cicco CE, et al. A switch from canonical to noncanonical Wnt signaling mediates drug resistance in colon cancer cells. PLoS One 2011;6:e27308

Discussion and Conclusion

Improving the treatment outcome for glioblastoma has emerged as a central goal in neurooncology over the past years. Compared to many other tumors, glioblastoma therapy has lacked major groundbreaking progresses to significantly improve the prognosis of newly diagnosed patients. Glioblastomas are characterized by an intratumoral heterogeneity, and present treatment concepts as chemotherapeutics and γ-irradiation fail to target the whole diversity of glioblastoma cells. Treatment failure and high frequency of tumor relapse is caused by the presence of GSCs, which are resistant to conventional therapy. Due to the infiltrative nature of glioblastomas and the high density of functional tissue in the brain that cannot be removed without severe damage, surgery alone is not sufficient. Therefore, novel therapeutic strategies targeting GSCs are urgently needed. The use of pharmacological inhibitors targeting pathways highly activated in GSCs is thought to be a promising approach to overcome treatment resistance in glioblastoma. One of these pathways is the Wnt pathway, whose inhibition is therefore an appealing treatment target for GSCs.

The first section of this thesis focuses on the question whether the small-molecule inhibitor LGK974 can directly target GSCs *in vitro*. It was first verified to what extend glioblastoma showed Wnt activation in surgical specimens. Positive nuclear staining for β-catenin was found in 19% of adult glioblastoma cases (8/43) and even 30% of all examined pediatric glioblastoma cases (9/30). However, staining was weak and only present in a small number of cells (77). Previous studies indicate Wnt being aberrantly activated through epigenetically silenced intrinsic Wnt inhibitors in more than 40% of glioblastomas (69). Another study found 28% of glioblastoma with positive immunohistological staining for cytoplasm-nuclear β-catenin (80). Taken this and previous data together, it could conceivably be hypothesized that every forth glioblastoma shows aberrant Wnt activation. One might wonder whether it makes sense to target Wnt signaling in glioblastoma, when only activated in less than 30% of cases. Undoubtedly, it is necessary to identify potential responders out of all

glioblastoma patients. For this, precise molecular markers indicating positive treatment response are indispensable and should be a focus of future research.

Before assessing functional effects of LGK974 treatment in cell culture, it was necessary to clarify whether LGK974 is reaching its target side. Inhibition of canonical Wnt signaling was detected by using a highly sensitive luciferase-based reporter system, showing pathway suppression in a dose dependent manner. On the contrary, mutant active β-catenin increased luciferase signaling in two cell lines tremendously. Suppression of the noncanonical Wnt pathway was not assessed due to the lack of a reliable readout (77). Studies from other research groups and the underlying mechanism of function suggest LGK974 to also target noncanonical Wnt signaling (81).

Next, glioblastoma cells were treated with LGK974 cultivated in stem cell media. Under LGK974 treatment, glioblastoma cells were impaired in their proliferation and viability, and apoptosis rate was increased in one cell line. Additionally, reduced expression of stem cell marker CD133 was detected in all cell lines, and stemness marker NANOG was decreased in GBM1 upon LGK974 treatment. CD133 positive cells presented to have higher canonical Wnt activity measured by the luciferase-based reporter system. Enhancement of differentiation marker expression was observed after treating cells with LGK974, with GFAP expression being increased in all cell lines and MAP2 expression being upregulated in LN229. Clonogenicity was reduced upon LGK974 treatment in stem cell media cultivated cell lines. These results indicate LGK974 to effectively reduce stemness in glioblastoma *in vitro* (77). Therefore, LGK974 can be regarded as a potential treatment option for glioblastoma by both targeting GSCs on one side, as well as inhibiting tumor progression in general on the other side.

In this study, Wnt activation indicated by present nuclear β-catenin and Axin2 expression was correlated to decreased overall survival (77). In contrast to earlier findings, however, no significance was detected. Shi et al. found nuclear accumulation of β-catenin to be associated with higher grading and worse prognosis in glioma (82). Looking at protein and mRNA expression levels, Denysenko et al. found Wnt3a, β-catenin and Wnt-associated transcription

factor 4 to be associated with histopathological tumor grading and worse prognosis in human glioma (83). Axin2 has not been characterized as a tumor marker for brain tumors so far, whereas correlation between Axin2 expression and tumor grading has been described for solid tumor entities such as colon, liver and breast cancer (84,85). In this study, mRNA expression levels of Axin2 were positively correlated to the amount of intranuclear β-catenin measured by a reporter assay. The results of this study indicate Axin2 as a marker for Wnt activation in glioblastoma. While being a direct target of β-catenin binding to LEF/TCF sides, the amount of Axin2 enables precise information about the level of canonical Wnt activation (86). As a member of the β-catenin destruction complex, Axin functions as a negative regulator of canonical Wnt, and Axin2 was observed to be positively correlated with the amount of nuclear β-catenin before (87). Nevertheless, this study did not investigate any thresholds, and therefore the role of Axin2 as a marker for Wnt activation in glioblastoma can only be stated with caution. Furthermore, Axin2 expression was not correlated with IC_{50} doses of LGK974, indicating that Axin2 is not a marker for therapeutic response to porcupine inhibition. Axin2 serves only as a readout for canonical Wnt activation, missing representation of noncanonical Wnt signaling, which could explain this discrepancy. Moreover, Axin2 expression levels of primary tumor samples were related to the expression levels of glioblastoma cell lines used in this study, thus it was guaranteed that a confident model for glioblastoma's biology was set up (77).

For the first time, this work gives detailed insight into the effects of pharmacological Wnt inhibiton in glioblastoma cells *in vitro*. The small molecule inhibitor LGK974, created by Novartis, was used in this study. It is directly targeting porcupine and therefore inhibiting both the canonical and the noncanonical branch of Wnt signaling pathway. As compared to colon carcinoma, Wnt activation in gliomas is rarely defined by somatic mutations, making upstream Wnt inhibitors a feasible therapeutic option. LGK974 is currently used in a phase I study for solid tumors such as colon carcinoma and pancreatic cancer. It is the first pharmacological Wnt inhibitor that was tested in patients. In 2016, at least 19 clinical trials used pharmacological Wnt inhibitors for treating cancer, but none was performed in gliomas to date (88).

Nevertheless, instead of monotherapy we rather suggest combinatorial therapy for the use of Wnt inhibitors. A key trend in cancer research and therapy observed these days is leaving monotherapy and increasing systematic treatment with several agents simultaneously to overcome treatment resistance in tumors. Therefore, the second paper included in this work attempts to assess whether the combinatorial therapy of LGK974 with TMZ or γ-irradiation harbors a synergistic effect and could be implicated as future therapy for glioblastoma. To answer this question, two cell lines defined by a methylated or unmethylated MGMT promoter, respectively, were used to evaluate if synergistic effects are MGMT methylation dependent. MGMT promoter methylation is the most important predictive biomarker for response to TMZ. Cell lines harboring an unmethylated MGMT promoter showed higher IC_{50} doses for TMZ (78).

This study shows that LGK974 works synergistically with TMZ and γ-irradiation, meaning the total effect of both treatments is greater than the sum of individual effects of each single treatment. This was the case for both MGMT methylated and unmethylated cell lines. On the basis of these results, it is possible to conclude that LGK974 sensitizes glioblastoma cells to chemo- and radiotherapy. This makes pharmacological Wnt inhibition a feasible additional therapy for current standard of care treatments. For combination of LGK974 and TMZ, synergy was stronger when applying lower dosages of both drugs. For clinical application, this could result in reduced side effects with no setback in effectiveness when treating patients with less amounts of anticancer agents. Lower dosages of LGK974 and TMZ are showing high efficacy when applied together *in vitro*, therefore it is possible that this can also be repeated while treating patients (78).

In the first part of the study higher dosages of LGK974 were used compared to the following investigations on combinatorial treatment (5µM vs. 1µM for GBM1, 5µM vs. 3µM for JHH520), since different incubation times were implemented. While applying LGK974 as a single agent, the cell lines were incubated with the drug for either 1 or 2 days. When treating the cells in combination with TMZ, greatest efficacy of TMZ was observed after 6 or 8 days, therefore a later time point was chosen as readout. Incubating the cells for a longer period with

LGK974 (while changing media with fresh drug every 2 days) changed individual IC_{50} doses to a lesser extent.

In this study the expression of ALDH3A1 was found to be downregulated upon LGK974 treatment. While applying LGK974 and TMZ in combination, an even greater extent of reduction in ALDH3A1 expression was achieved. These results suggest ALDH3A1 to play a role in resistance against TMZ, since combined LGK974 and TMZ was also observed to have synergistic effects on suppressing cell viability. ALDH3A1's role in chemoresistance was further investigated in this study, since ALDH3A1 was the gene mostly deregulated upon combination treatment of LGK974 and TMZ. Therefore, the protein was genetically blocked in glioblastoma cell lines. ALDH3A1 knock-down cells showed reduced cell viability in general, and more reduction of cell viability under TMZ treatment compared to control cells. These results suggest reduction in ALDH3A1 to sensitize glioblastoma cells towards chemotherapy. It is unfortunate that this study does not include data on genetically overexpressed ALDH3A1 showing increased resistance upon TMZ treatment; therefore, it is not possible to claim that ALDH3A1 increases chemoresistance in glioblastoma. Also, there was no correlation between ALDH3A1 expression levels and TMZ IC_{50} doses in our cell lines observed. Therefore, mechanisms beside ALDH3A1 regulation have to be involved in TMZ resistance in glioblastoma cell lines. Nevertheless, this study confirms that decreased ALDH3A1 expression levels lead to increased sensitivity towards TMZ in glioblastoma *in vitro* (78).

In contrast, treating ALDH3A1 knock-down cells with LGK974 was not more effective than same treatment in control cells, implicating that ALDH3A1 might be regulated through Wnt signaling. This hypothesis matches to previous research, where a link between Wnt signaling and ALDH3A1 expression has been drawn already. In hepatocellular carcinoma, ALDH3A1 is associated with intranuclear β-catenin expression assessed by immunohistological staining (89). Another group could show that breast cancer cells with high stemness character are increased in their β-catenin, Wnt-1 and ALDH3A1 expression levels at the same time (90). However, there was no correlation between ALDH3A1 expression and intranuclear β-catenin measured by a reporter assay construct

found in this study, indicating that there is no direct linkage between ALDH3A1 and Wnt in the cell models being used. Canonical Wnt signaling was reduced upon TMZ and LGK974 treatment, similar to the results for ALDH3A1 protein and mRNA expression observed throughout same treatment conditions. Moreover, ALDH3A1 expression was reduced in GBM1 β-catenin knock-down cells (78). These results implicate a positive correlation between ALDH3A1 expression and canonical Wnt activation. Additionally, two promoter binding sides for TCF in the ALDH3A1 promoter region were found. Taken together, it can be assumed that ALDH3A1 is at least partly regulated though Wnt signaling.

The results of this study support previous research in cancer which links ALDH3A1 expression to resistance against chemotherapy. In head and neck squamous cell carcinoma, induction of ALDH3A1 with the small molecule activator Alda-89 increased cell survival under cisplatin (91). In breast cancer cells, ALDH3A1 expression was linked to resistance against four different chemotherapeutics (4-hydroxyperoxycyclophosphamide, doxorubicin, etoposide, and 5-fluorouracil) and γ-irradiation. In contrast to the results from this study, the group observed decreased clonogenic potential as well as decreased expression of stem cell markers such as Sox2 in ALDH3A1 overexpressing cells, whereas our results indicate this tendency for ALDH3A1 knock-down cells (92). Regarding other publications investigating ALDH3A1's role in disease, the general sense matches with the observation made in this study, for ALDH3A1 having a proliferation promoting character (93). These findings may support the hypothesis that ALDH3A1 could be a biological marker for prediction of a tumor's response towards chemotherapy with TMZ. A note of caution is due here since it was not tested whether upregulated ALDH3A1 expression is correlated to resistance against TMZ, nor exist any clinical data on ALDH3A1 expression and tumor progression in glioblastoma. Results from researchers working on different tumor entities suggest a potential role for ALDH3A1 as a biomarker predicting chemoresistance. Data on Burkitt lymphoma show that ALDH3A1 is mutated in tumor relapse of patients that underwent chemotherapy (94). In hormone-receptor positive early breast cancer, single nucleotide polymorphisms (SNPs) in ALDH3A1 are correlated

with poor overall survival (95). Wu et al. stained 93 gastric tumor specimens, revealing ALDH3A1 expression correlates with dysplasia, lymph node metastases and cancer stage, and therefore suggest ALDH3A1 as a marker for stemness in gastric cancer (96). ALDH3A1 was already suggested to mark somatic stem cells as well as CSCs, like hematopoetic stem cells (97), prostate CSCs (98), and breast CSCs (92). This study presents data showing reduction of stem cell markers Nestin, Sox2 and CD133 (only in one cell line) in ALDH3A1 knock-down cells as well as reduced clonogenicity (in two cell lines) (78). In line with previous data on ALDH3A1 and stem cell character, we infer ALDH3A1 to might serve as a marker for stemness in GSCs. Nevertheless, this data is solely based on four glioblastoma cell models, impeding generalization to all glioblastoma patients. Analyzing a larger cohort of tissue specimens with referring to clinical data is indispensable for stating ALDH3A1 as a marker for glioma stem cells and a proposal for future research projects.

ALDH3A1 belongs to a family of 19 so far different aldehyde dehydrogenases. These enzymes regulate the oxygenation of aldehydes with the use of either NAD or NADP as a coenzyme, being involved in detoxification of metabolites, drugs and alcohol. Over the years aldehyde dehydrogenases were found to have numerous functions in physiological and pathological processes. Nine aldehyde dehydrogenases were described to show higher activity in somatic and cancer stem cells including GSCs, most notably ALDH1A1 and ALDH3A1. ALDH1A3 was found to be enriched in mesenchymal GSCs from high grade gliomas (99). Zhang et al. found that ALDH1A3 promoter methylation leading to less protein expression was correlated to better outcome for glioblastoma patients lacking IDH mutation and MGMT promoter hypermethylation (100). These results suggest other members of the ALDH family to also play a role in glioma maintenance, worsening clinical prognosis.

ALDH3A1 is located on chromosome 17 in humans and best known for its role in corneal epithelial homeostasis. In contrast to other aldehyde dehydrogenases, it is only poorly represented in human liver cells (101). As ALDHs, and especially ALDH3A1, harbor an essential role in abrogating oxidative stress, ALDH3A1 is highly expressed in organs constantly exposed to environmental stimuli, including cells in lung, stomach, cornea and skin. High

expression of ALDH3A1 was linked to increased drug resistance and proliferation rates before, as well as resistance against aldehydes derived from lipid peroxidation. Since ALDH3A1 was already known for its role in chemoresistance and cancer stem cells, several small molecule inhibitors have already been developed and tested *in vitro*, whereas *in vivo* experiments have not been yet performed (102–104). Sensitization towards chemotherapy was observed among application of ALDH3A1 inhibitors, also in glioblastoma cell lines (103). In breast cancer cells, ALDH3A1 leads to increased resistance against irradiation (92), indicating that ALDH3A1-inhibition might also be supportive among radiotherapy in cancer.

Finally, the question remains as to whether Wnt or ALDH3A1 acts as better therapeutic target. Both Wnt signaling and ALDH3A1 contribute to stemness as well as chemoresistance in cancer. Inhibiting compounds are also available for both targets. When it comes to fully genetically Wnt blocked mice, the animals show a high mortality at early embryonic time points, indicating the importance of Wnt signaling in embryonic development (105). Mice lacking Tcf7/L2 receptor or genetic blockade of β-catenin were impaired in self-renewal of crypts located in the intestine (106). Since Wnt signaling is known for its importance in somatic stem cells, pharmacological blockage might cause severe side effects on highly self-renewing cells. This indicates a small therapeutic index, comparable to most chemotherapeutic drugs. In contrast, ALDH3A1 knockout mice only suffer from eye cataracts, but are similar to control animals regarding growth and survival time (107). To fully answer the question whether Wnt or ALDH3A1 inhibition is the better target, more research using controlled trials for LGK974 and TMZ combination as well as ALDH3A1 inhibitors tested at least in xenograft models are indispensable.

A research paper published recently in Cancer Cell concerning systemic administration of LGK974 in mice (5 mg/kg twice a day), described good tolerance of the applied dose. Interestingly, the group found LGK974 to cross the BBB and to significantly improve survival in glioma xenografts when combined with TMZ *in vivo*. LGK974 treatment alone showed a trend of improved survival without significance (108). The data suggests LGK974 to be a better treatment when combined with TMZ. This paper supplements our *in vitro*

results with *in vivo* data and strongly supports the hypothesis of LGK974 being a potential treatment for glioblastoma patients in combination with TMZ.

Since this study was limited to the use of LGK974, it is not possible to generalize our *in vitro* results to every other pharmacological Wnt inhibitor. LGK974 is supposed to inhibit both canonical and noncanonical Wnt signaling equally, whereas most other pharmacological compounds only target canonical Wnt signaling. This makes it even more difficult to transfer the results of this study to other pharmacological Wnt inhibitors. To further characterize pharmacological Wnt inhibition in glioblastoma in general, the work was enriched by a third paper, listing all pharmacological compounds targeting Wnt and used in preclinical studies in gliomas. The review paper gives an overview about mechanism and target side of each compound, as well as its stage of research in glioblastoma and in cancer in general. Furthermore, common alterations of Wnt signaling in glioblastoma, as well as prognostic markers linked to Wnt signaling are listed (79). Unfortunately, no marker has been described to predict effective pharmacological Wnt inhibition in glioblastoma so far. In pancreatic adenocarcinomas and colorectal cancer without *APC* mutations, genetic alterations of *RNF43* were shown to predict sensitivity towards compounds inhibiting Wnt secretion such as LGK974 (75,109). Identification of predictive markers for glioblastoma patients benefiting from anti-Wnt therapy is indispensable for clinical application and should be subject of prospective research. In conclusion, the third paper illustrates clinical perspectives on Wnt-targeted therapies in glioblastoma, mentioning estimated obstacles and suggesting implication for future investigations (79). It is worth noting that the review was published in 2016 and novel Wnt inhibitors were designed and new clinical studies enrolled since then. Even so, none of the compounds listed in the review or any other pharmacological Wnt inhibitor has made it into clinical studies for glioblastoma so far.

As a conclusion, this work confirms pharmacological Wnt inhibition to be useful as a potential therapy for glioblastoma by directly targeting GSCs and reducing tumor growth *in vitro*. Furthermore, LGK974 works synergistically with TMZ treatment *in vitro,* at least partly mediated through the enzyme ALDH3A1, which

was identified as another potential therapeutic target for sensitizing glioblastoma cells to TMZ. These results are highly relevant for clinical attempts to target GSCs in glioblastoma. A natural progression of these data would be performing *in vivo* experiments with ALDH3A1 inhibitors in glioblastoma xenograft models and setting up a clinical trial evaluating the effect of combined TMZ and LGK974 treatment for glioblastoma patients.

References

1. Virchow R. Zwanzig Vorlesungen gehalten während der Monate Februar, März und April 1858 im pathologischen Institut zu Berlin. Berlin: First edition. August Hirschwald; 1858. Die Cellularpathologie in ihrer Begründung auf physiologische and pathologische Gewebelehre.

2. von Deimling A, Herausgeber. Gliomas [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 2009 [zitiert 10. Februar 2018]. (Recent Results in Cancer Research; Bd. 171). Verfügbar unter: http://link.springer.com/10.1007/978-3-540-31206-2

3. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, u. a. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. Acta Neuropathol. Juni 2016;131(6):803–20.

4. Ohgaki H, Kleihues P. The definition of primary and secondary glioblastoma. Clin Cancer Res. 15. Februar 2013;19(4):764–72.

5. Cairncross JG, Ueki K, Zlatescu MC, Lisle DK, Finkelstein DM, Hammond RR, u. a. Specific genetic predictors of chemotherapeutic response and survival in patients with anaplastic oligodendrogliomas. J Natl Cancer Inst. 7. Oktober 1998;90(19):1473–9.

6. Smith JS, Perry A, Borell TJ, Lee HK, O'Fallon J, Hosek SM, u. a. Alterations of chromosome arms 1p and 19q as predictors of survival in oligodendrogliomas, astrocytomas, and mixed oligoastrocytomas. J Clin Oncol. Februar 2000;18(3):636–45.

7. Intergroup Radiation Therapy Oncology Group Trial 9402, Cairncross G, Berkey B, Shaw E, Jenkins R, Scheithauer B, u. a. Phase III trial of chemotherapy plus radiotherapy compared with radiotherapy alone for pure and mixed anaplastic oligodendroglioma: Intergroup Radiation Therapy Oncology Group Trial 9402. J Clin Oncol. 20. Juni 2006;24(18):2707–14.

8. Liu X-Y, Gerges N, Korshunov A, Sabha N, Khuong-Quang D-A, Fontebasso AM, u. a. Frequent ATRX mutations and loss of expression in adult diffuse astrocytic tumors carrying IDH1/IDH2 and TP53 mutations. Acta Neuropathol. November 2012;124(5):615–25.

9. Sahm F, Reuss D, Koelsche C, Capper D, Schittenhelm J, Heim S, u. a. Farewell to oligoastrocytoma: in situ molecular genetics favor classification as either oligodendroglioma or astrocytoma. Acta Neuropathol. Oktober 2014;128(4):551–9.

10. Ishii N, Tada M, Hamou MF, Janzer RC, Meagher-Villemure K, Wiestler OD, u. a. Cells with TP53 mutations in low grade astrocytic tumors evolve clonally to malignancy and are an unfavorable prognostic factor. Oncogene. 21. Oktober 1999;18(43):5870–8.

11. Wiestler B, Capper D, Holland-Letz T, Korshunov A, von Deimling A, Pfister SM, u. a. ATRX loss refines the classification of anaplastic gliomas and identifies a subgroup of IDH mutant astrocytic tumors with better prognosis. Acta Neuropathol. September 2013;126(3):443–51.

12. Yuen BTK, Knoepfler PS. Histone H3.3 Mutations: A Variant Path to Cancer. Cancer Cell. November 2013;24(5):567–74.

13. Verhaak RGW, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, u. a. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. Cancer Cell. 19. Januar 2010;17(1):98–110.

14. Larjavaara S, Mäntylä R, Salminen T, Haapasalo H, Raitanen J, Jääskeläinen J, u. a. Incidence of gliomas by anatomic location. Neuro-Oncology. 1. Juli 2007;9(3):319–25.

15. Steed TC, Treiber JM, Patel K, Ramakrishnan V, Merk A, Smith AR, u. a. Differential localization of glioblastoma subtype: implications on glioblastoma pathogenesis. Oncotarget. 3. Mai 2016;7(18):24899–907.

16. Heimberger AB, Hlatky R, Suki D, Yang D, Weinberg J, Gilbert M, u. a. Prognostic effect of epidermal growth factor receptor and EGFRvIII in glioblastoma multiforme patients. Clin Cancer Res. 15. Februar 2005;11(4):1462–6.

17. Nikolaev S, Santoni F, Garieri M, Makrythanasis P, Falconnet E, Guipponi M, u. a. Extrachromosomal driver mutations in glioblastoma and low-grade glioma. Nature Communications. 4. Dezember 2014;5:5690.

18. James CD, Carlbom E, Dumanski JP, Hansen M, Nordenskjold M, Collins VP, u. a. Clonal genomic alterations in glioma malignancy stages. Cancer Res. 1. Oktober 1988;48(19):5546–51.

19. Fults D, Tippets RH, Thomas GA, Nakamura Y, White R. Loss of heterozygosity for loci on chromosome 17p in human malignant astrocytoma. Cancer Res. 1. Dezember 1989;49(23):6572–7.

20. Johnson DR, O'Neill BP. Glioblastoma survival in the United States before and during the temozolomide era. Journal of Neuro-Oncology. April 2012;107(2):359–64.

21. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJB, u. a. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med. 10. März 2005;352(10):987–96.

22. Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, u. a. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N Engl J Med. 9. November 2000;343(19):1350–4.

23. Hegi ME, Diserens A-C, Gorlia T, Hamou M-F, de Tribolet N, Weller M, u. a. *MGMT* Gene Silencing and Benefit from Temozolomide in Glioblastoma. New England Journal of Medicine. 10. März 2005;352(10):997–1003.

24. Westphal M, Hilt DC, Bortey E, Delavault P, Olivares R, Warnke PC, u. a. A phase 3 trial of local chemotherapy with biodegradable carmustine (BCNU) wafers (Gliadel wafers) in patients with primary malignant glioma. Neuro-oncology. 2003;5(2):79–88.

25. Brem H, Piantadosi S, Burger PC, Walker M, Selker R, Vick NA, u. a. Placebo-controlled trial of safety and efficacy of intraoperative controlled delivery by biodegradable

polymers of chemotherapy for recurrent gliomas. The Polymer-brain Tumor Treatment Group. Lancet. 22. April 1995;345(8956):1008–12.

26. Vogelbaum MA, Aghi MK. Convection-enhanced delivery for the treatment of glioblastoma. Neuro-oncology. März 2015;17 Suppl 2:ii3–8.

27. Stupp R, Taillibert S, Kanner AA, Kesari S, Steinberg DM, Toms SA, u. a. Maintenance Therapy With Tumor-Treating Fields Plus Temozolomide vs Temozolomide Alone for Glioblastoma: A Randomized Clinical Trial. JAMA. 15. Dezember 2015;314(23):2535–43.

28. Weller M, Butowski N, Tran DD, Recht LD, Lim M, Hirte H, u. a. Rindopepimut with temozolomide for patients with newly diagnosed, EGFRvIII-expressing glioblastoma (ACT IV): a randomised, double-blind, international phase 3 trial. Lancet Oncol. Oktober 2017;18(10):1373–85.

29. Liau LM, Ashkan K, Tran DD, Campian JL, Trusheim JE, Cobbs CS, u. a. First results on survival from a large Phase 3 clinical trial of an autologous dendritic cell vaccine in newly diagnosed glioblastoma. J Transl Med. 29. Mai 2018;16(1):142.

30. Wen P, Reardon D, Phuphanich S, Aiken R, Landolfi J, Curry W, u. a. AT-60 * A RANDOMIZED DOUBLE BLIND PLACEBO-CONTROLLED PHASE 2 TRIAL OF DENDRITIC CELL (DC) VACCINE ICT-107 FOLLOWING STANDARD TREATMENT IN NEWLY DIAGNOSED PATIENTS WITH GBM. Neuro-Oncology. 1. November 2014;16(suppl 5):v22–v22.

31. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, u. a. Improved Survival with Ipilimumab in Patients with Metastatic Melanoma. New England Journal of Medicine. 19. August 2010;363(8):711–23.

32. ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 200 0 Feb 29 - . Identifier NCT02337491, Pembrolizumab +/- Bevacizumab for Recurrent GBM; 2015, Jan 13 [cited 2018 Aug 09]; [about 6 screens]. Available from: https://clinicaltrials.gov/ct2/show/results/NCT02337491?id=NCT02337491&rank=1&load=cart.

33. ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 200 0 Feb 29 - . Identifier NCT02336165, Phase 2 Study of MEDI4736 in Patients With Glioblastoma; 2015 Jan 12 [cited 2018 Aug 09]; [about 8 screens]. Available from: https://clinicaltrials.gov/ct2/show/NCT02336165.

34. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, u. a. Identification of human brain tumour initiating cells. Nature. 18. November 2004;432(7015):396–401.

35. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, u. a. Identification of a cancer stem cell in human brain tumors. Cancer Res. 15. September 2003;63(18):5821–8.

36. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, u. a. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature. 17. Februar 1994;367(6464):645–8.

37. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. Juli 1997;3(7):730–7.

38. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proceedings of the National Academy of Sciences. 1. April 2003;100(7):3983–8.

39. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature. 19. November 2006;445:106.

40. Prince ME, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P, u. a. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proceedings of the National Academy of Sciences. 16. Januar 2007;104(3):973–8.

41. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, u. a. Identification of pancreatic cancer stem cells. Cancer Res. 1. Februar 2007;67(3):1030–7.

42. Eramo A, Lotti F, Sette G, Pilozzi E, Biffoni M, Di Virgilio A, u. a. Identification and expansion of the tumorigenic lung cancer stem cell population. Cell Death & Differentiation. März 2008;15(3):504–14.

43. Yang ZF, Ho DW, Ng MN, Lau CK, Yu WC, Ngai P, u. a. Significance of CD90+ Cancer Stem Cells in Human Liver Cancer. Cancer Cell. Februar 2008;13(2):153–66.

44. Schatton T, Murphy GF, Frank NY, Yamaura K, Waaga-Gasser AM, Gasser M, u. a. Identification of cells initiating human melanomas. Nature. 17. Januar 2008;451(7176):345–9.

45. Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV, u. a. Direct isolation of human central nervous system stem cells. Proc Natl Acad Sci USA. 19. Dezember 2000;97(26):14720–5.

46. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, u. a. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature. 7. Dezember 2006;444(7120):756–60.

47. Salmaggi A, Boiardi A, Gelati M, Russo A, Calatozzolo C, Ciusani E, u. a. Glioblastoma-derived tumorospheres identify a population of tumor stem-like cells with angiogenic potential and enhanced multidrug resistance phenotype. Glia. Dezember 2006;54(8):850–60.

48. Liu G, Yuan X, Zeng Z, Tunici P, Ng H, Abdulkadir IR, u. a. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. Mol Cancer. 2. Dezember 2006;5:67.

49. Rich JN. Cancer stem cells in radiation resistance. Cancer Res. 1. Oktober 2007;67(19):8980–4.

50. Auffinger B, Tobias AL, Han Y, Lee G, Guo D, Dey M, u. a. Conversion of differentiated cancer cells into cancer stem-like cells in a glioblastoma model after primary chemotherapy. Cell Death Differ. Juli 2014;21(7):1119–31.

51. Robey E. Notch in vertebrates. Curr Opin Genet Dev. August 1997;7(4):551–7.

52. Egloff AM, Grandis JR. Molecular pathways: context-dependent approaches to Notch targeting as cancer therapy. Clin Cancer Res. 1. Oktober 2012;18(19):5188–95.

53. Olsauskas-Kuprys R, Zlobin A, Osipo C. Gamma secretase inhibitors of Notch signaling. Onco Targets Ther. 2013;6:943–55.

54. Fan X, Khaki L, Zhu TS, Soules ME, Talsma CE, Gul N, u. a. NOTCH pathway blockade depletes CD133-positive glioblastoma cells and inhibits growth of tumor neurospheres and xenografts. Stem Cells. Januar 2010;28(1):5–16.

55. Xu R, Shimizu F, Hovinga K, Beal K, Karimi S, Droms L, u. a. Molecular and Clinical Effects of Notch Inhibition in Glioma Patients: A Phase 0/I Trial. Clin Cancer Res. 1. Oktober 2016;22(19):4786–96.

56. Munoz JL, Rodriguez-Cruz V, Walker ND, Greco SJ, Rameshwar P. Temozolomide resistance and tumor recurrence: Halting the Hedgehog. Cancer Cell Microenviron. 2015;2(2).

57. Xu Q, Yuan X, Liu G, Black KL, Yu JS. Hedgehog signaling regulates brain tumor-initiating cell proliferation and portends shorter survival for patients with PTEN-coexpressing glioblastomas. Stem Cells. Dezember 2008;26(12):3018–26.

58. ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 200 0 Feb 29 - . Identifier NCT00980343, GDC-0449 in Treating Patients With Recurrent Glioblastoma Multiforme That Can Be Removed by Surgery; 2009 Sep 21 [cited 2018 Aug 09]; [about 9 screens]. Available from: https://clinicaltrials.gov/ct2/show/NCT00980343.

59. Rimkus T, Carpenter R, Qasem S, Chan M, Lo H-W. Targeting the Sonic Hedgehog Signaling Pathway: Review of Smoothened and GLI Inhibitors. Cancers. 15. Februar 2016;8(2):22.

60. Filbin MG, Dabral SK, Pazyra-Murphy MF, Ramkissoon S, Kung AL, Pak E, u. a. Coordinate activation of Shh and PI3K signaling in PTEN-deficient glioblastoma: new therapeutic opportunities. Nat Med. November 2013;19(11):1518–23.

61. Koul D. PTEN signaling pathways in glioblastoma. Cancer Biol Ther. September 2008;7(9):1321–5.

62. ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 200 0 Feb 29 - . Identifier NCT01339052, Phase II Study of BKM120 for Subjects With Recurrent Glioblastoma; 2011 Apr 20 [cited 2018 Aug 09]; [about 9 screens]. Available from: https://clinicaltrials.gov/ct2/show/NCT01339052.

63. ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 200 0 Feb 29 - . Identifier NCT00590954, Clinical and Molecular-Metabolic Phase II Trial of Perifosine for Recurrent/Progressive Malignant Gliomas; 2008 Jan 11 [cited 2018 Aug 09]; [about 5 screens]. Available from: https://clinicaltrials.gov/ct2/show/NCT00590954.

64. Francipane MG, Lagasse E. mTOR pathway in colorectal cancer: an update. Oncotarget. 15. Januar 2014;5(1):49–66.

65. Ciruelos Gil EM. Targeting the PI3K/AKT/mTOR pathway in estrogen receptor-positive breast cancer. Cancer Treat Rev. August 2014;40(7):862–71.

66. Fumarola C, Bonelli MA, Petronini PG, Alfieri RR. Targeting PI3K/AKT/mTOR pathway in non small cell lung cancer. Biochem Pharmacol. 1. August 2014;90(3):197–207.

67. Li X, Wu C, Chen N, Gu H, Yen A, Cao L, u. a. PI3K/Akt/mTOR signaling pathway and targeted therapy for glioblastoma. Oncotarget. 31. Mai 2016;7(22):33440–50.

68. Jauliac S, López-Rodriguez C, Shaw LM, Brown LF, Rao A, Toker A. The role of NFAT transcription factors in integrin-mediated carcinoma invasion. Nat Cell Biol. Juli 2002;4(7):540–4.

69. Götze S, Wolter M, Reifenberger G, Müller O, Sievers S. Frequent promoter hypermethylation of Wnt pathway inhibitor genes in malignant astrocytic gliomas. Int J Cancer. 1. Juni 2010;126(11):2584–93.

70. Takahashi-Yanaga F, Kahn M. Targeting Wnt Signaling: Can We Safely Eradicate Cancer Stem Cells? Clinical Cancer Research. 15. Juni 2010;16(12):3153–62.

71. Wickström M, Dyberg C, Milosevic J, Einvik C, Calero R, Sveinbjörnsson B, u. a. Wnt/β-catenin pathway regulates MGMT gene expression in cancer and inhibition of Wnt signalling prevents chemoresistance. Nat Commun. 25. November 2015;6:8904.

72. Zhang H, Luo H, Hu Z, Peng J, Jiang Z, Song T, u. a. Targeting WISP1 to sensitize esophageal squamous cell carcinoma to irradiation. Oncotarget [Internet]. 20. März 2015 [zitiert 26. Januar 2018];6(8). Verfügbar unter: http://www.oncotarget.com/fulltext/3358

73. Cojoc M, Peitzsch C, Kurth I, Trautmann F, Kunz-Schughart LA, Telegeev GD, u. a. Aldehyde Dehydrogenase Is Regulated by β-Catenin/TCF and Promotes Radioresistance in Prostate Cancer Progenitor Cells. Cancer Res. 1. April 2015;75(7):1482–94.

74. Liu J, Pan S, Hsieh MH, Ng N, Sun F, Wang T, u. a. Targeting Wnt-driven cancer through the inhibition of Porcupine by LGK974. Proc Natl Acad Sci USA. 10. Dezember 2013;110(50):20224–9.

75. Jiang X, Hao H-X, Growney JD, Woolfenden S, Bottiglio C, Ng N, u. a. Inactivating mutations of RNF43 confer Wnt dependency in pancreatic ductal adenocarcinoma. Proc Natl Acad Sci USA. 30. Juli 2013;110(31):12649–54.

76. ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 200 0 Feb 29 - . Identifier NCT01351103, A Study of LGK974 in Patients With Malignancies Dependent on Wnt Ligands; 2011 May 10 [cited 2018 Aug 09]; [about 8 screens]. Available from: https://clinicaltrials.gov/ct2/show/NCT01351103.

77. Kahlert UD, Suwala AK, Koch K, Natsumeda M, Orr BA, Hayashi M, u. a. Pharmacologic Wnt Inhibition Reduces Proliferation, Survival, and Clonogenicity of Glioblastoma Cells. J Neuropathol Exp Neurol. September 2015;74(9):889–900.

78. Suwala AK, Koch K, Rios DH, Aretz P, Uhlmann C, Ogorek I, u. a. Inhibition of Wnt/beta-catenin signaling downregulates expression of aldehyde dehydrogenase isoform 3A1 (ALDH3A1) to reduce resistance against temozolomide in glioblastoma *in vitro*.

Oncotarget [Internet]. 27. April 2018 [zitiert 12. Dezember 2018];9(32). Verfügbar unter: http://www.oncotarget.com/fulltext/25210

79. Suwala AK, Hanaford A, Kahlert UD, Maciaczyk J. Clipping the Wings of Glioblastoma: Modulation of WNT as a Novel Therapeutic Strategy. Journal of Neuropathology & Experimental Neurology. Mai 2016;75(5):388–96.

80. Liu C, Tu Y, Sun X, Jiang J, Jin X, Bo X, u. a. Wnt/beta-Catenin pathway in human glioma: expression pattern and clinical/prognostic correlations. Clin Exp Med. Juni 2011;11(2):105–12.

81. Voloshanenko O, Schwartz U, Kranz D, Rauscher B, Linnebacher M, Augustin I, u. a. β-catenin-independent regulation of Wnt target genes by RoR2 and ATF2/ATF4 in colon cancer cells. Scientific Reports [Internet]. Dezember 2018 [zitiert 2. Mai 2018];8(1). Verfügbar unter: http://www.nature.com/articles/s41598-018-20641-5

82. Shi Z, Qian X, Li L, Zhang J, Zhu S, Zhu J, u. a. Nuclear translocation of β-catenin is essential for glioma cell survival. J Neuroimmune Pharmacol. Dezember 2012;7(4):892–903.

83. Denysenko T, Annovazzi L, Cassoni P, Melcarne A, Mellai M, Schiffer D. WNT/β-catenin Signaling Pathway and Downstream Modulators in Low- and High-grade Glioma. Cancer Genomics Proteomics. Februar 2016;13(1):31–45.

84. Gabrovska PN, Smith RA, Tiang T, Weinstein SR, Haupt LM, Griffiths LR. Development of an eight gene expression profile implicating human breast tumours of all grade. Mol Biol Rep. April 2012;39(4):3879–92.

85. Lustig B, Jerchow B, Sachs M, Weiler S, Pietsch T, Karsten U, u. a. Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. Mol Cell Biol. Februar 2002;22(4):1184–93.

86. Jho E -h., Zhang T, Domon C, Joo C-K, Freund J-N, Costantini F. Wnt/ -Catenin/Tcf Signaling Induces the Transcription of Axin2, a Negative Regulator of the Signaling Pathway. Molecular and Cellular Biology. 15. Februar 2002;22(4):1172–83.

87. Leung JY, Kolligs FT, Wu R, Zhai Y, Kuick R, Hanash S, u. a. Activation of AXIN2 expression by beta-catenin-T cell factor. A feedback repressor pathway regulating Wnt signaling. J Biol Chem. 14. Juni 2002;277(24):21657–65.

88. Lu B, Green B, Farr J, Lopes F, Van Raay T. Wnt Drug Discovery: Weaving Through the Screens, Patents and Clinical Trials. Cancers. 1. September 2016;8(9):82.

89. Calderaro J, Nault J-C, Bioulac-Sage P, Laurent A, Blanc J-F, Decaens T, u. a. ALDH3A1 is overexpressed in a subset of hepatocellular carcinoma characterised by activation of the Wnt/ß-catenin pathway. Virchows Archiv. Januar 2014;464(1):53–60.

90. Zhang F, Song C, Ma Y, Tang L, Xu Y, Wang H. Effect of fibroblasts on breast cancer cell mammosphere formation and regulation of stem cell-related gene expression. Int J Mol Med. September 2011;28(3):365–71.

91. Kim J, Shin JH, Chen C-H, Cruz L, Farnebo L, Yang J, u. a. Targeting aldehyde dehydrogenase activity in head and neck squamous cell carcinoma with a novel small molecule inhibitor. Oncotarget. 8. August 2017;8(32):52345–56.

92. Voulgaridou G-P, Kiziridou M, Mantso T, Chlichlia K, Galanis A, Koukourakis MI, u. a. Aldehyde dehydrogenase 3A1 promotes multi-modality resistance and alters gene expression profile in human breast adenocarcinoma MCF-7 cells. Int J Biochem Cell Biol. August 2016;77(Pt A):120–8.

93. Xu X, Chai S, Wang P, Zhang C, Yang Y, Yang Y, u. a. Aldehyde dehydrogenases and cancer stem cells. Cancer Letters. Dezember 2015;369(1):50–7.

94. Wever CM, Geoffrion D, Grande BM, Yu S, Alcaide M, Lemaire M, u. a. The genomic landscape of two Burkitt lymphoma cases and derived cell lines: comparison between primary and relapse samples. Leuk Lymphoma. 3. Januar 2018;1–16.

95. Kuo S-H, Yang S-Y, You S-L, Lien H-C, Lin C-H, Lin P-H, u. a. Polymorphisms of ESR1, UGT1A1, HCN1, MAP3K1 and CYP2B6 are associated with the prognosis of hormone receptor-positive early breast cancer. Oncotarget. 2. Februar 2017;

96. Wu D, Mou Y-P, Chen K, Cai J-Q, Zhou Y-C, Pan Y, u. a. Aldehyde dehydrogenase 3A1 is robustly upregulated in gastric cancer stem-like cells and associated with tumorigenesis. Int J Oncol. August 2016;49(2):611–22.

97. Gasparetto M, Sekulovic S, Brocker C, Tang P, Zakaryan A, Xiang P, u. a. Aldehyde dehydrogenases are regulators of hematopoietic stem cell numbers and B-cell development. Exp Hematol. April 2012;40(4):318-329.e2.

98. Yan J, De Melo J, Cutz J-C, Aziz T, Tang D. Aldehyde dehydrogenase 3A1 associates with prostate tumorigenesis. Br J Cancer. 13. Mai 2014;110(10):2593–603.

99. Mao P, Joshi K, Li J, Kim S-H, Li P, Santana-Santos L, u. a. Mesenchymal glioma stem cells are maintained by activated glycolytic metabolism involving aldehyde dehydrogenase 1A3. Proc Natl Acad Sci USA. 21. Mai 2013;110(21):8644–9.

100. Zhang W, Yan W, You G, Bao Z, Wang Y, Liu Y, u. a. Genome-wide DNA methylation profiling identifies ALDH1A3 promoter methylation as a prognostic predictor in G-CIMP-primary glioblastoma. Cancer Lett. 1. Januar 2013;328(1):120–5.

101. Muzio G, Maggiora M, Paiuzzi E, Oraldi M, Canuto RA. Aldehyde dehydrogenases and cell proliferation. Free Radical Biology and Medicine. Februar 2012;52(4):735–46.

102. Parajuli B, Kimble-Hill AC, Khanna M, Ivanova Y, Meroueh S, Hurley TD. Discovery of novel regulators of aldehyde dehydrogenase isoenzymes. Chem Biol Interact. 30. Mai 2011;191(1–3):153–8.

103. Parajuli B, Fishel ML, Hurley TD. Selective ALDH3A1 inhibition by benzimidazole analogues increase mafosfamide sensitivity in cancer cells. J Med Chem. 23. Januar 2014;57(2):449–61.

104. Parajuli B, Georgiadis TM, Fishel ML, Hurley TD. Development of selective inhibitors for human aldehyde dehydrogenase 3A1 (ALDH3A1) for the enhancement of cyclophosphamide cytotoxicity. Chembiochem. 21. März 2014;15(5):701–12.

105. van Amerongen R, Berns A. Knockout mouse models to study Wnt signal transduction. Trends Genet. Dezember 2006;22(12):678–89.

106. Schepers A, Clevers H. Wnt signaling, stem cells, and cancer of the gastrointestinal tract. Cold Spring Harb Perspect Biol. 1. April 2012;4(4):a007989.

107. Lassen N, Bateman JB, Estey T, Kuszak JR, Nees DW, Piatigorsky J, u. a. Multiple and additive functions of ALDH3A1 and ALDH1A1: cataract phenotype and ocular oxidative damage in Aldh3a1(-/-)/Aldh1a1(-/-) knock-out mice. J Biol Chem. 31. August 2007;282(35):25668–76.

108. Griveau A, Seano G, Shelton SJ, Kupp R, Jahangiri A, Obernier K, u. a. A Glial Signature and Wnt7 Signaling Regulate Glioma-Vascular Interactions and Tumor Microenvironment. Cancer Cell. 6. April 2018;

109. van de Wetering M, Francies HE, Francis JM, Bounova G, Iorio F, Pronk A, u. a. Prospective derivation of a living organoid biobank of colorectal cancer patients. Cell. 7. Mai 2015;161(4):933–45.

Supplementary data

Kahlert, UD., Suwala, AK., Koch, K., Natsumeda, M., Orr, BA., Hayashi,M., Maciaczyk, J., Eberhart, CG. (2015), Pharmacological WNT inhibition reduces proliferation, survival and clonogenicity of GBM cells. *Journal of Neuropathology & Experimental Neurology*, (Volume 74), pages 889–900

GBM1

Loci	
AMEL	X,Y
CSF1PO	11,12
D13S317	8,11
D16S539	8,13
D21S11	29,30.2
D5S818	10,11
D7S820	10
TH01	6,7
TPOX	8
vWA	16,17

JHH520

Loci	
AMEL	X
CSF1PO	11
D13S317	11,12
D16S539	9,13
D21S11	29
D5S818	11
D7S820	10,11
TH01	9,10
TPOX	8
vWA	19

LN229

Loci	
AMEL	X
CSF1PO	12
D13S317	10,11
D16S539	12
D21S11	29,30
D5S818	11,12
D7S820	8,11
TH01	9.3
TPOX	8
vWA	16,19

GBM10

Loci	
AMEL	X,Y
CSF1PO	11
D13S317	8,12
D16S539	13
D21S11	29,30
D5S818	10
D7S820	7,10
TH01	6
TPOX	8,9
vWA	17

U87NS

Loci	
AMEL	X
CSF1PO	10,11
D13S317	8,11
D16S539	12
D21S11	28,32.2
D5S818	11,12
D7S820	8,9
TH01	9.3
TPOX	8
vWA	15,17

Supporting Information S1. Short tandem repeat profiles of cell cultures used for functional studies.

primers

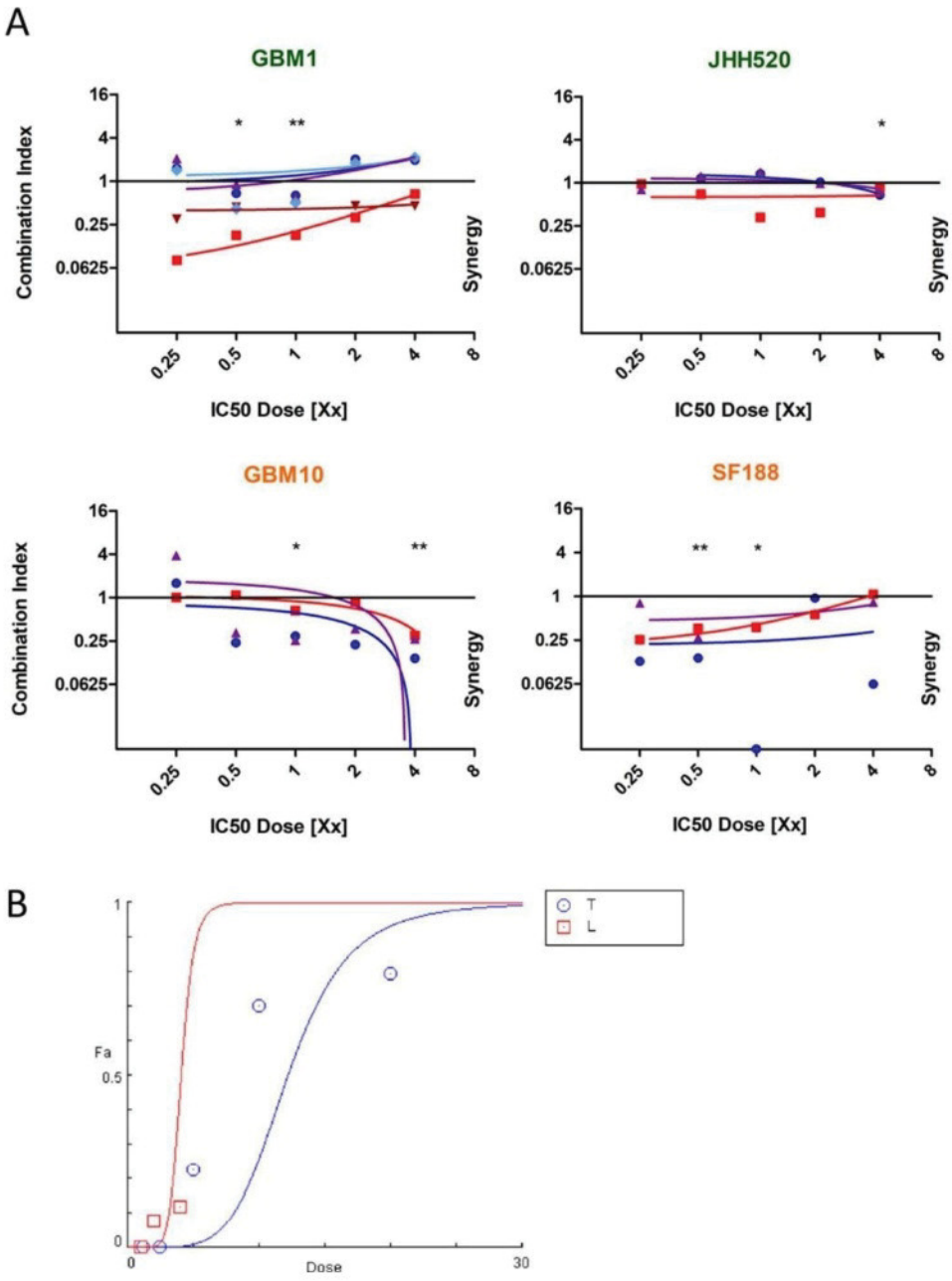
gene name	sequences
beta ACTIN	AGCACAATGAAGATCAA
	CGATCCACACGGAGTACTTG
AXIN2	AGCCAAAGCGATCTACAAAAGG
	GGTAGGCATTTTCCTCCATCAC
NANOG	TGATTTGTGGGCCTGAAGAAAA
	GAGGCATCTCAGCAGAAGACA

antibodies

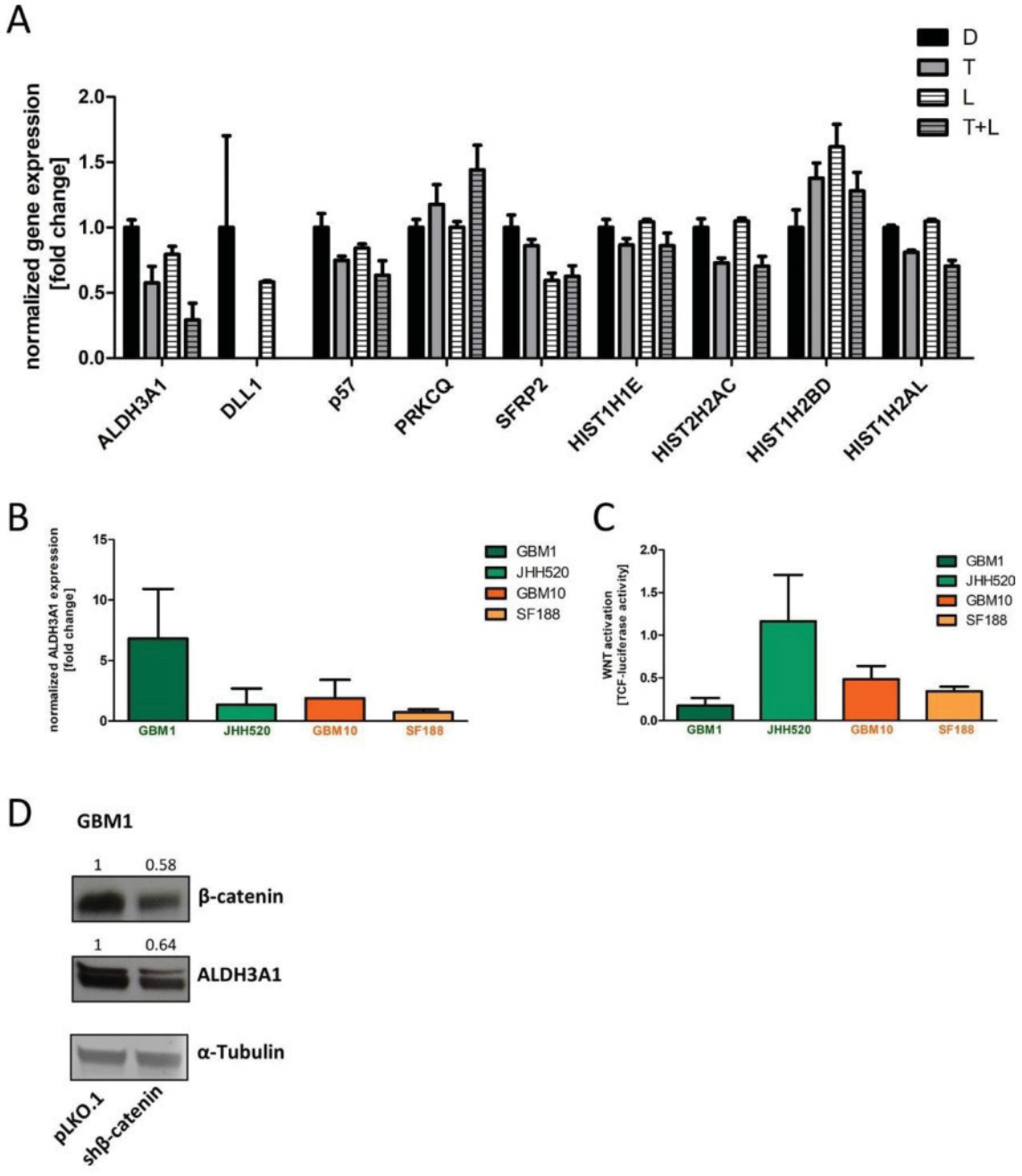
antigen	product	dilution for Western blot
GFAP Clone 6F2	#M0761, Dako	1 to 3000
GAPDH Clone 6C5	#Sc-32233, Santa Cruz	1 to 1000
MAP2 Clone H-300	#Sc-20172, Santa Cruz	1 to 2000

Supplemental Data S2. Primer sequences and antibody specifications for Western blot-based protein analyses.

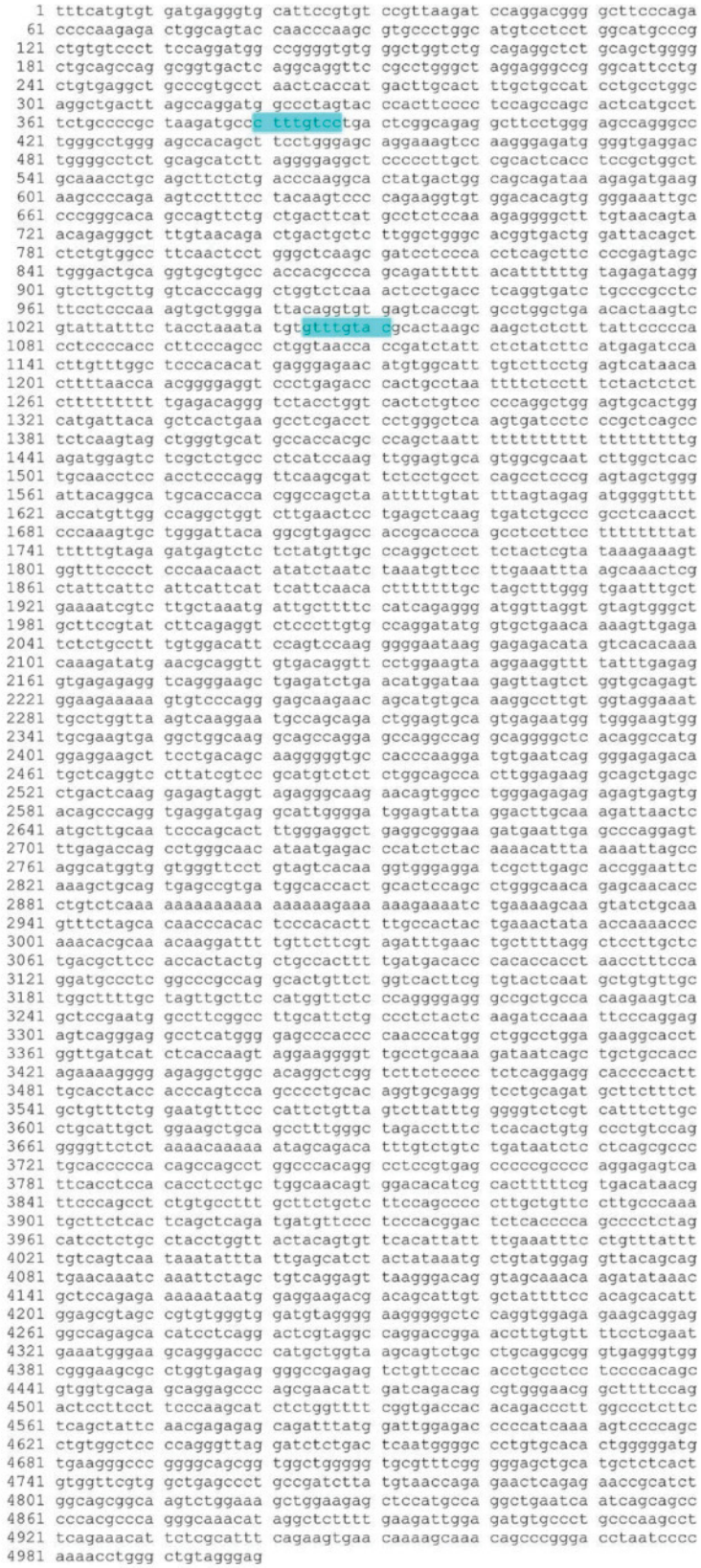
Suwala AK., Koch K., Herrera Rios D., Aretz P., Uhlmann C., Ogorek I., Felsberg J., Reifenberger G., Köhrer K., Deenen R., Steiger HJ., Kahlert UD., Maciaczyk J., (2018), Inhibition of Wnt/beta-catenin signaling downregulates expression of aldehyde dehydrogenase isoform 3A1 (ALDH3A1) to reduce resistance against temozolomide in glioblastoma *in vitro*. *Oncotarget*, (Volume 9), pages 22703 – 22716



Supplementary Figure 1: (A) LGK974 acts synergistically in combination with γ-irradiation. The x-axis represents different multiples of the detected IC_{50} dose for each treatment and cell line. The axis of ordinates demonstrates the calculated combination index. The combination index is calculated based on the median-effect equation, taking each value from γ-irradiation, LGK974 single treatment and combination of both treatments for one specific dose into account. In one experiment, the combination index is calculated for five different doses and represented by one colored line. If the combination index is less than 1, both treatments act synergistically. If it is equal 1, both treatments act additively. If the combination index is more than 1, the effects are antagonistic. Each color represents one independent experiment (GBM1 $n = 5$ independent experiments; JHH520, GBM10 and SF188 $n = 3$ independent experiments). MGMT methylated cell lines presented in green, MGMT unmethylated cell lines presented in orange. (B) Dose-effect curve of TMZ and LGK974 in GBM1. Effect (Fa) is represented on the axis of ordinates; dose is represented on the x-axis on linear scales. D: DMSO; T: TMZ; I: γ-irradiation; L: LGK974.



Supplementary Figure 2: (**A**) mRNA expression of 9 genes showing most pronounced differential expression in LGK974 and TMZ treated GBM1 cells as detected by microarray analysis. (**B**) Basal ALDH3A1 mRNA expression of all four cell lines. (**C**) Basal Wnt activation of all four cell lines assessed by T cell factor (TCF) luciferase reporters. (**D**) ALDH3A1 protein expression is reduced in GBM1 β-catenin-knock-down cells. pLKO.1 serves as the vector control. Data is presented as mean ± standard deviation (SD). D: DMSO; T: TMZ; L: LGK974.



Supplementary Figure 3: Genomic sequence on chromosome 17 (nucleotides 19753433-19748433) with binding sides for TCF in der *ALDH3A1* promoter marked in blue. The sequence was retrieved from NCBI GenBank.

Supplementary Table 1: Primer sequences used for qPCR analyses (A) and oligonucleotides used as RNA-targeting sequences for CRISPR/Cas9-mediated knock-down of ALDH3A1

A

Primer	fwd	rev
β2-microgobulin	GTTGCTCCACAGGTAGCTCTAG	ACAAGCTTTGAGTGCAAGAGATTG
ALDH3A1	TGTTCTCCAGCAACGACAAG	CTGACCTTCAGGCCTTCATC
P57	GCTGAACGCCGAGGACCAGAACCG	CGGGCACCGAGTCGCTGTCCACTT
DLL1	TGGCGCAGGCATCGA	GGCGGCTGATGAGTCTTTCT
PRKCQ	CTCCCTTATATGGCCCCTGG	CTTCTGCGATGCCACTGTAC
SFRP2	TTCCCCAAGCACACTCCTAG	TACAAGATTCGGGTGGGCTT
HIST1H1E	CCAAGAAGAGCGCCAAGAAG	CGCCTTGGGTTTAACTGCTT
HIST2H2AC	GTGGCAAACAAGGAGGCAAG	GTCTTCTTGTTGTCCCGAGC
HIST1H2BD	ACCGGCACCTTGATCTTGTA	GGCTGGGGAGTAAAGAGTGT
HIST1H2AL	GACAACAAGAAGACCCGCAT	CTCGGTCTTCTTGGGCAGTA
Sox2	TGGACAGTTACGCGCACAT	CGAGTAGGACATGCTGTAGGT
Nestin	GGCGCACCTCAAGATGTCC	CTTGGGGTCCTGAAAGCTG

B

Oligo	fwd	rev
Control	CACCGGGTGAACCGCATCGAGCTGA	AAACTCAGCTCGATGCGGTTCACCC
crALDH3A1	CACCGTTCGACCATATCCTGTACAC	AAACGTGTACAGGATATGGTCGAAC
crALDH3A1 2nd	CACCGGGACACCCCCATTGATTACT	AAACAGTAATCAATGGGGGTGTCCC

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