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WNT/β-Catenin-vermittelte Resistenz gegenüber Glukose-Entzug in Glioblastom-Stammzellen

WNT/β-Catenin-mediated resistance to glucose deprivation in glioblastoma stem-like cells

Dissertation

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Widmung

Waxaan mahad gaar ah u jeedinayaa hooyaday, oo ah saaxiibadeeyda ugu fiican, ku dayashadayda, magangalkayga, iyo qalbigayga. Hooyada iyo gurigu weligood waa hal, weligoodna mid waa guri iyo hooyo. Labada erey waxay isku mid yihiin afkeenna hooyo, waana sax. Iyada la'aanteed tani ma suurta gasheen.
Mahad aan dhammaad lahayn ayaa iska leh hooyaday.

Mahadsanid hooyo

Besonderer Dank gilt meiner Mutter, die zugleich meine beste Freundin, mein Vorbild, meine Zuflucht und mein Herz ist. Die Mutter und das Zuhause sind für immer eins, und für immer eins sind das Zuhause und die Mutter. Synonym sind die beiden Begriffe in unserer Muttersprache und zwar zu Recht. Ohne sie wäre dies nicht ansatzweise möglich.
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I. Zusammenfassung

Das Isocitrat-Dehydrogenase (IDH)-Wildtyp-Glioblastom ist ein primärer Hirntumor, der sich durch seine hohe Malignität und der damit einhergehenden schlechten Prognose trotz Ausreizung sämtlicher Therapiemöglichkeiten auszeichnet. Die mediane Gesamtüberlebensdauer beträgt 15 Monate bei Patienten, die sich einer supramarginalen Resektion der Tumormasse und einer simultanen, kombinierten Radiochemotherapie unterziehen. Die Malignität des IDH-Wildtyp-Glioblastoms ist unter anderem auf eine schnell proliferierende Population von Zellen zurückzuführen, die die Fähigkeit zur asymmetrischen Zellteilung besitzen und somit zum Selbsterhalt des Tumors beitragen. Diese auch als Tumorstammzellen bezeichneten Zellen sind unter extremen Stoffwechselbedingungen äußerst anpassungs- und -überlebensfähig. Aus diesem Grund war es das Ziel der vorliegenden Arbeit, die reziproke Regulation des WNT-Signalweges (Wingless und Int1-Proteins) in nährstofflimitierten Mikroumgebungen als phylogenetisch hoch konservierten Stammzellsignalweg in Hinblick auf die Vermittlung metabolischer Plastizität und Adaptationsfähigkeit hin untersuchen. *In-vitro*-Kulturen von Glioblastomzelllinien (GSZ) wurden in Standard-Zellkulturglukosekonzentrationen (450mg/dL) und in abnehmenden Glukosekonzentrationen bis hin zum Glukoseentzug zu verschiedenen Zeitpunkten (24, 48 und 72 Stunden) kultiviert. Glukoseentzug förderte das Überleben einer kleinen Population „starvationresistenter“ und WNT-hochregulierender GSZ, erhöhte die Klonogenität und Invasion dieser Zellen und ihre Chemosensitivität für bestimmte Chemotherapeutika, wie z. B. Pharmaka, die den WNT-Singalweg inhibieren (LGK974 & Berberine). Darüber hinaus kam es zu einer verstärkten Expression einiger WNT-Zielgene wie *ZEB1* und *AXIN2*. Die alleinige Applikation von LGK974 oder dessen Gabe in Kombination mit Glukoseentzug veränderte maßgeblich die intrazelluläre Metabolitenkonzentrationen der GSZ im Vergleich zu GSZ, die unter Standardzellkulturbedingungen kultiviert wurden, was für eine WNT-spezifische oder -mediierte metabolische Adaptabilität dieser Zellen spricht. Die eigenen Ergebnisse deuten somit auf eine WNT-vermittelte metabolische Plastizität und Überlebensfähigkeit von GSZ in nährstoffbegrenzten Mikroumgebungen hin. Für diese Arbeit an etablierten Tumorstammzelllinien lagen gültige Ethikvoten (5841R, 2019-484) vor.

II. Abstract

Isocitrate dehydrogenase (IDH)-wildtype glioblastoma is a primary brain tumor that is marked by its high aggressiveness and poor prognosis, with an overall median survival of 15 months in individuals who receive supramarginal surgical tumor resection followed by combined radiochemotherapy. The malignancy of IDH-wildtype glioblastoma is caused by a rapidly proliferating population of cells with the ability to self-renewal, survival, and adaptability to harsh microenvironmental conditions. For this reason, the work summarized in this thesis investigated the reciprocal regulation of the WNT-pathway as a phylogenetically well-conserved stemness-pathway with its distinctive implications in promoting WNT-mediated ability of metabolic plasticity and adjustment to nutrient-restricted environments in glioblastoma. Glioblastoma cancer stem-like cells (GSC) were cultured in conventional cell-culture glucose concentrations (450 mg/dL) as well as in decreasing glucose concentrations for various time points (24, 48, and 72 hours (h)). Glucose deprivation promoted the survival of a small population of starvation-resistant and WNT-upregulated GSC, enhanced clonogenicity and invasion of these cells, and improved chemosensitivity to certain chemotherapeutics, such as pharmaceuticals that inhibit the WNT-pathway like LGK974 and Berberine. Glucose deprivation also caused increased expression of WNT-target genes such as *ZEB1* and *AXIN2*. LGK974 application, alone or in conjunction with glucose deficiency, dramatically modified metabolite concentrations in intracellular compartments, indicating WNT-mediated metabolic modulation. These findings show that WNT-specific or -mediated metabolic flexibility plays a role in GBM-CSC survival under nutrient-limited microenvironmental conditions. This work on established tumor stem cell lines was supported by valid ethics votes (5841R, 2019-484).

III. Abkürzungsverzeichnis

5-ALA: 5-Aminolevulinate

Acetyl-CoA: Acetyl coenzyme A

Akt: synonym to Protein kinase B (PKB) Ak strain transforming

AMPK: AMP-activated protein kinase

APC: Adenomatous polyposis coli protein

ATP: Adenosine triphosphate

Axin2: Axis inhibition protein 2

BCL: B-cell lymphoma protein

BSA: Bovine Serum Albumin

c-Myc: C-myelocytomatosis oncogene product

C°: Celsius

Ca^{2+}: Calcium

CAM: Cell adhesion molecules

CBP: CAMP responsive element binding protein binding protein

CCD26: Coiled-coil domain containing 26 (long non-coding RNA)

CCL2: CC-chemokine ligand 2

cCT: Cranial computer tomography

CD133: AC133, synonym to Prominin 1 (PROM1)

CD24: Cluster of differentiation 24 or heat stable antigen CD24 (HSA)

CD4+: Cluster of differentiation 4

CD44: Cluster of differentiation 44

CD8+: Cluster of differentiation 8

Cdc42: Cell division control protein 42 homolog

CDKN2A: Cyclin-dependent kinase inhibitor 2A

CDKN2B: Cyclin dependent kinase inhibitor 2B

cDNA: Complementary DNA

cGMP: Cyclic guanosine monophosphate

CK1α: Casein kinase α

cMRI: Cranial magnetic resonance imaging

CNS: Central nervous system;

conc.: Concentration

CREB: CAMP responsive element binding protein

CSC: Cancer stem-like cells

CSF: Cerebrospinal fluid

CT: Computer tomography

ctrl: Control (standard glucose concentration)

d: Deci

d1- : Day 1 of glucose depletion

d2-: Day 2 of glucose depletion

DAG: Diacylglycerol

DAPI: 4′,6-Diamidino-2-phenylindole

DC: Detergent compatible

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

Dsh: Dishevelled

E-Cadherin: Epithelial cadherin

ECM: Extracellular matrix component

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

EMT: Epithelial-mesenchymal-transition

EMT TF: Epithelial-mesenchymal-transition transcription factor

ERK: Extracellular signal-regulated kinase

FADH2: Flavin adenine dinucleotide

FBS: Fetal bovine serum

FGF: Fibroblast growth factor

Fwd: Forward

Fz: Frizzled

G-protein: Guanine nucleotide-binding proteins

g: Gram

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GC-MS: Gas chromatography mass spectrometry

Glc-: Depleted glucose (0 mg/dL)

Glc+: Standard glucose concentration (450 mg/dL)

GLUT1: Glucose unilateral transporter 1

GLUT3: Glucose unilateral transporter 3

GSC: Glioblastoma stem cell

GSK3: Glycogen synthase kinase 3

GSZ: Glioblastomstammzelle

Gy: Gray

h: Hour(s)

H3 K27: Histone 3, lysine (K) position 27

HDAC: Histone deacetylase

HIF1α: Hypoxia-inducible factor 1-alpha

HMGCS2: 3-Hydroxymethylglutaryl-CoA synthase 2

HNPCC: Non-polyposis colon carcinoma

IDH1: Isocitrate dehydrogenase 1

IGF-1: Insulin-like growth factor 1

IL-35: Interleukin-35

JNK: Jun N-terminal kinase

KEGG: Kyoto Encyclopedia of Genes and Genomes

Ki-67: Proliferation marker Ki-67 (Ki is derived from Kiel, well 67)

L: Liter

L1CAM1: L1 cell adhesion molecule 1

LDH-A: Lactate dehydrogenase A

LRP: Lipoprotein-related receptors

M-MLV: Moloney murine leukemia virus

m: Milli or meter

M: Molar

mAB: Monoclonal antibody

MCT-1: Monocarboxylate transporter 1

MGMT: O6-methylguanine-DNA-methyltransferase

min: Minute(s)

miRNA: Micro RNA

MLH1: MutL protein homolog 1 (mismatch repair protein MLH1)

mmHg: Millimeter of mercury

MMTV: Murine mammary tumor virus

MRI: Magnetic resonance imaging

mRNA: Messenger RNA

MSAB: Methyl 3-[(4-methylphenyl)sulfonyl]amino-benzoate

MSH2: MutS homolog 2 (mismatch repair protein MSH2)

MSH6: MutS homolog 6 (mismatch repair protein MSH6)

mTOR: Mammalian Target of Rapamycin

mTORC1: Mammalian target of rapamycin complex 1 or mechanistic target of rapamycin complex 1

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;

MXI2: A mitogen-activated protein kinase

***n*:** Sample size

n: Nano

NADPH: Nicotinamide adenine dinucleotide phosphate

NANOG: Tír na nÓg (Irish: "Land of the Young")

NBT: Nitro blue tetrazolium chloride

NEAA: Non-essential amino acids

NF1: Neurofibromin

NFkB: Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells

NGS: Next generation sequencing

NPC: Neural precursor cell

NSC: Neural stem cell

NRH1: Neurotrophin receptor homolog 1

OCT4: Octamer-binding transcription factor 4

OPC: Olidogendrocyte precursor cell

***p*-value:** Probability value

p: Petite/ the shorter arm of the chromosome

p: pico

p53/ TP53/TRP53: Transformation-related protein 53 (53 kilodalton)

PBS: Phosphate buffered saline

PDE: Phosphodiesterase

PDH: Pyruvate dehydrogenase

PDK1: Pyruvate dehydrogenase kinase 1

PFA: Paraformaldehyde

pH: Potential of hydrogen

PHLDB1: Pleckstrin homology like domain family B member 1

PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

PKC: Protein kinase C

PKM2: Pyruvate kinase M2

PLC: Phospholipase C

PLK1: Polo-like kinase-1

PMS2: PMS1 homolog 2

PORCN: Porcupine

PP2A: Protein phosphatase 2A

PRPP: Phosphoribosyl pyrophosphate

PTEN: Phosphatase and tensin homolog

PTK7: Protein-tyrosine kinase 7

PYGO: Pygopus protein

q: The larger arm of the chromosome (chosen as following letter in alphabet after p)

Rac1: Ras-related C3 botulinum toxin substrate 1

Rev: Reverse

Rhoa: Ras homolog family member A

RIPA: Radioimmunoprecipitation Assay

RNA: Ribonucleic acid

ROR2: Receptor tyrosine kinase-like orphan receptor 2

ROS: Reactive oxygen species

rpm: Rounds per minute

RT-qPCR: Real-time quantitative PCR

RT: Room temperature

RTEL1: Regulator of telomere length 1

SD: Standard deviation

Ser: Serine

SFRP: Secreted frizzled-related proteins

SLC1A1: Solute carrier family 1 member 1

SLC1A5: Solute carrier family 1 member 5; synonym to ASCT2: Alanine, Serine, Cysteine Transporter 2

SNAIL: Zinc finger protein SNAI1

SOX2: Sex determining region Y-box 2

SREBf1: Sterol regulatory element-binding transcription factor 1

SREBP1-1: Sterol regulatory element-binding protein 1

SRSF: Serine and arginine-rich splicing factor

T1: Longitudinal relaxation time

T2: Transverse relaxation time

TAM: Tumor-associated macrophages

TBST: Tris-buffered saline containing Tween

TCA: Tricarboxylic acid

TCF/LEF: T cell factor/lymphoid enhancer factor family

TERT: Telomerase reverse transcriptase

TF: Tissue factor

TF: Transcription factor

Thr: Threonine

TME: Tumor microenvironment

TMZ: Temozolomide

Tregs: Regulatory T cells

VEGF: Vascular endothelial growth factor

WHO: World Health Organization

WNT: Wingless and Int-1

WNT1: Int1

ZEB1: Zinc finger E-box binding homeobox 1

β-catenin: Beta-catenin

µ: Micro

IV. Table of Contents

1. Introduction

The most prevalent primary malignant brain tumors are gliomas [2]. The World Health Organization (WHO) classifies malignancies of the central nervous system (CNS) into various tumor types based on the combination of histological characteristics and molecular biomarkers [3]. The WHO classification distinguishes three primary tumor entities among diffusely infiltrating gliomas in adults: IDH-mutant astrocytomas of CNS WHO grades 2, 3, or 4, IDH-mutant and 1p/19q-codeleted oligodendrogliomas of CNS WHO grades 2 or 3, and IDH-wildtype glioblastomas of CNS WHO grade 4. A subpopulation of tumor cells with stem-like characteristics, such as the capacity for self-renewal and adaptation to extreme and unfavorable microenvironmental conditions in terms of nutrient availability, are thought to be the primary drivers of malignant glioma growth and resistance to therapy [4]–[7]. Tumor growth induces hypovascularized, hypoxic, and nutrient-deprived tumor areas, which favor the survival of cell subpopulations that are resistant to nutrient stress [8].

A promising field of study that may explain mechanisms contributing to tumor cell survival is the metabolic plasticity and reprogramming of tumor cells in nutrient-limited microenvironments in IDH-wildtype glioblastoma [9]. Glioblastoma stemness is crucial for fostering the durability, self-renewal, and metabolic adaptability of glioblastoma cells, which have been linked to stem-like tumor cells in glioblastomas [10].

In multiple forms of cancer, particularly breast cancer, colorectal cancer, prostate cancer, and glioblastoma, several stemness factors and phylogenetically conserved stemness pathways, such as the WNT pathway, have been implicated as drivers of cancer cell stemness and tumor growth [11]. During embryogenesis and organogenesis, WNT signaling has pleiotropic effects on cell physiology and development, particularly in neural tissue, as well as on maintaining metabolic homeostasis. The WNT/β-catenin - dependent (also known as the canonical WNT pathway) and non-canonical pathways are two examples of the independent WNT signaling pathways.

The planar cell polarity pathway and the WNT/Calcium(Ca^{2+}) pathway are further divisions of the non-canonical pathway, each of which promotes different cellular effects by activating various downstream cascades. The classical WNT signaling route is the most studied pathway and governs the abundance of the transcriptional co-activator β-catenin and regulates important developmental events [12]. In many different tumor forms, including glioblastoma, altered β-catenin signaling, particularly via the aberrant activation of the classical WNT pathway, plays a significant role in encouraging tumor growth [13]. For example, CC-chemokine ligand 2 (CCL2) and β-catenin have been shown to increase monocyte migration toward glioma cells [14]. In addition, WNT-mediated β-catenin signaling encourages metabolic alterations in cancer [15]. For instance, it has been demonstrated that the transcriptional upregulation of the monocarboxylate-transporter 1 (MCT-1) is mediated by β-catenin binding to TCF/LEF sites, which activates the promoter and, in turn, increases aerobic glycolysis and lactate secretion [16]. The sodium-dependent unspecific amino acid transporter (SLC1A5), the pyruvate dehydrogenase (PDH), the pyruvate kinase M2 (PKM2), the lactate dehydrogenase A (LDH-A), and the glucose transporter 1(GLUT1) have all been shown to be upregulated when the conventional WNT pathway is activated [15].

The Akt-mTOR pathway is activated by non-canonical WNT signaling, which also enhances glycolysis [14]. By mTORC1 and β-catenin signaling, fatty acid oxidative metabolism is also enhanced [17]. Augmentation of the mTOR-pathway leads to an upregulated pentose phosphate bypass by an increased expression of glucose-6-phosphate dehydrogenase. It thus supplies increasing amounts of reduction-equivalents, such as the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH/H+), and the provision of phosphoribosyl pyrophosphate (PRPP) for subsequent purine and pyrimidine synthesis [18].

The work summarized in this thesis examined the effects of glucose concentration on the cellular metabolism and biological characteristics of GSCs *in vitro* in this study, concentrating on cellular models of IDH-wildtype GSCs. The effects of glucose restriction on the WNT/β-catenin pathway in glioblastoma was examined as a proof of concept and to test the impact of pharmacological WNT inhibition on GSCs under glucose-restricted circumstances.

1.1. Tumors of the central nervous system

The Latin term "tumor" primarily refers to space-occupying processes of any kind. However, the term is usually equated with space-demanding processes of neoplastic origin [19]. Intracranial space-occupying processes need not necessarily be of neoplastic etiology. Examples of non-neoplastic space-occupying processes are processes such as hemorrhagic, post-ischemic lesions with secondary hemorrhagic components, cerebrospinal fluid resorption disorders, such as hygroma, tissue pressure increase of (post)-inflammatory genesis or caused by electrolyte imbalances and/or pH disturbances, intoxications or trauma [20]–[24].

As aforementioned, the term tumor is widely used to refer to neoplastic processes, which are characterized by autonomous and pathological tissue proliferation [25]. These neoplasms may be of primary or secondary origin. As this thesis addresses brain tumors, it will elaborate on the more detailed classification of brain tumors. Primary brain tumors arise from the brain's own or adjacent tissue, with malignant primary brain tumors being overall less common than benign primary brain tumors [26]–[28]. Secondary brain tumors correspond to intracerebral or intraspinal metastases of primary tumors located outside the central nervous system [26]. Most often they originate from primaries located in tissues or organs drained by the vena cava and thus very often metastasize to the brain [29]–[31]. Brain metastases are often seen in advanced stages of breast cancer, lung cancer, and malignant melanoma [32], and less commonly in lymphoma [33], genitourinary cancers [34], [35], as well as proximal and distal gastrointestinal-tumors [34].

Neoplasms are also distinguished with regard to their malignancy. Malignancy is determined by certain characteristics of a tumor, such as growth, differentiation, cell alteration, and clinical course [36]–[38]. The last criterion in particular requires special attention because histopathological benign tumors can be classified as (semi-)malignant due to a clinically unfavorable course. A well-known example of this is basal cell carcinoma, which extremely rarely metastasizes, but grows locally destructive [39], [40].

Benign tumors of the central nervous system include most meningiomas, which can rarely succumb to malignant transformation [41], craniopharyngiomas, hemangioblastomas, gangliocytomas, and schwannomas [42], [43].
Examples of malignant brain tumors are neuroepithelial neoplasms such as gliomas, being the most prevalent primary malignant brain tumor entities, affecting children as well as adults [44].

1.2. Gliomas

The term glioma is a collective term for brain tumors whose tumor cells primarily resemble the glial cell type from a cytopathologic standpoint, but do not necessarily have to originate from mature glial cells [45]. Glial cells are cells of the nervous system that serve as a support scaffold for the neurons, but also provide electrical insulation and thus faster, saltatory transmission of stimuli, which occurs almost exclusively in vertebrates [46]. In addition, these cell types display an essential function in regulating metabolite/fluid exchange, pH and electrolyte homeostasis [47]–[49].

The following section will focus on the glial cells of the central nervous system. These are divided into macroglia and microglia. Macroglia include astrocytes, oligodendrocytes, and ependymal cells [50]. Quantitatively, astrocytic cells are the most abundant cell type in the mammalian central nervous system [51]. Astrocytes are star-shaped cells that form the blood-brain barrier [52] together with endothelial cells and pericytes [53]. The cell-cell interactions between these cell types are among the closest in the entire vertebrate organism [54]–[56]. Accordingly, astrocytes provide controlled fluid and electrolyte exchange and thus determine pH homeostasis [47]–[49]. In addition, they play an important role in information processing, as they also release excitatory transmitters such as glutamate into the synaptic cleft and thus can modulate information processing [57]. Furthermore, these cells are also responsible for the emergence of so-called glial scars in the context of regenerative processes [52], [58].

Oligodendrocytes are responsible for myelin formation in the central nervous system and sheath up to 50 axons. This sheathing leads to electrical insulation and saltatory

conduction of excitation. The amount of myelin formed is decisive for the speed of excitation conduction [59], [60].In addition, these cell types can secrete growth factors and promote neuronal growth [61].

Microglial cells are both glial cells of the central nervous system and mononuclear phagocytes. They are of special importance for the immune defense of the CNS. Furthermore, they are involved in regenerative processes by initiating inflammatory processes and display an essential function in the embryonic development of the nervous system [62].

Thus, the term glioma refers to the cytological similarity of the tumor cells to one of the different glial cell types but this is not to be confused with the origin as previously described [45]. Gliomas occur in the brain, spinal cord, and optic nerves [63]–[65] - The optic nerve, from an ontogenetic point of view, equates to a bulged-out part of the diencephalon, and thus the glial cells residing there are of central nervous (oligodendrocytic) origin [66], [67].

1.3. Classification of Gliomas

The term “Glioma” was first described in 1863 by Rudolf Virchow, who was also the first to describe glial cells [68], [69]. In 1884, Harvey Cushing and Percival Bailey defined the term glioma in more detail with appropriate terminology [70]. A biological gradation was created in 1949 by James Watson Kernohan. Klaus Joachim Zülch combined both the terminology of Cushing and Bailey and the biological grading of Kernohan and thus created the foundation for today's WHO classification [71].

The CNS WHO classification is a constantly updated and worldwide valid classification of brain tumors based on numerous criteria. The classification is based on tumor histology (tissue architecture, cell types) and molecular pathology (gene mutation, amplification, deletion, methylation, gene products, etc.). The malignancy of these entities is mainly derived from histological malignancy criteria. These evaluate the tumors for instance by the presence of cell nuclear atypia, mitotic activity, invasive growth, microvascular proliferation and necrosis.

In June 2021, the 5th edition of the CNS WHO classification was published. In this version, tumor types are more finely classified based on increasingly sophisticated molecular pathology. Another new feature is the grading within a tumor type, which now applies to various tumors (for instance IDH-mutant astrocytomas and meningiomas). In this version, the terms "type" and "subtype" are used instead of "entity" and "variant". To achieve comparability with other WHO grading systems, this version now employs Arabic numerals instead of Roman numerals for tumor grades, i.e. CNS WHO grades 1-4. Within the fifth edition of the CNS WHO classification, the following tumor groups are distinguished: gliomas, glioneural tumors, neuronal tumors, choroid plexus tumors, embryonal CNS tumors, pineal tumors, tumors of the cranial and spinal nerves, meningiomas, mesenchymal (non-meningeal) tumors, melanocytic tumors, hematolymphoid neoplasms, germ cell tumors, tumors of the sellar region, and CNS metastases.

New tumor types have been identified that were for instance previously assigned to be a subtype of another tumor type in the preceding version (for example, the diffuse hemispheric glioma, H3 G34-mutant was previously considered under the IDH-wildtype glioblastomas) [72]. A major change in the 5th edition of the CNS WHO classification has occurred within gliomas, glioneuronal, neuronal tumors, and also embryonal tumors.

This is because 14 new tumor entities have been differentiated within gliomas and glioneuronal tumors. In this new classification, diffuse gliomas are also divided into adult-type and pediatric-type tumors as a novel approach [73].

This thesis will focus on the classification of gliomas according to the current 5th edition of the CNS WHO classification. Here, the following tumor groups are distinguished under the heading of gliomas, glioneuronal and neuronal tumors: Adult-type diffuse gliomas, pediatric-type diffuse low-grade gliomas, pediatric-type diffuse high-grade gliomas, circumscribed astrocytic gliomas, glioneural and neuronal tumors, and ependymal tumors. It is within these tumor groups that most changes have occurred in the latest version of the CNS WHO classification. For the sake of simplicity, the following text will focus on the tumor types that are most important for this work, namely adult-type diffuse gliomas. Pediatric-type diffuse gliomas will not be discussed, as these tumor types are very dissimilar from adult-type diffuse gliomas [73]. Adult-type diffuse gliomas are further distinguished into astrocytomas with IDH mutation, oligodendrogliomas with IDH mutation and 1p/19q codeletion, and glioblastoma without IDH mutation [72].

Gliomas, glioneuronal tumors, and neuronal tumors	Adult-type diffuse gliomas • Astrocytoma, IDH-mutant • Oligodendroglioma, IDH-mutant and 1p/19q-codeleted • Glioblastoma, IDH-wildtype
	Pediatric-type diffuse low-grade gliomas
	Pediatric-type diffuse high-grade gliomas
	Circumscribed astrocytic gliomas
	Glioneuronal and neuronal tumors
	Ependymal tumors

Table 1: Overview of gliomas, glioneuronal tumors, and neuronal tumors according to the 5th edition of the CNS WHO classification 2021. Shown are the six distinct tumor groups listed under this header, with the individual tumor types shown only for the adult-type diffuse gliomas. Modified according to Louis et al. [3]**.**

1.3.1. Glioblastoma

The following paragraphs focus on glioblastoma IDH-wildtype, formerly also known as glioblastoma multiforme, however, this is no longer considered as a recommended term [74]. Traditional histological subtypes of glioblastoma are giant cell glioblastoma, gliosarcoma, and epithelioid glioblastoma [75].

Glioblastoma, IDH-wildtype is characterized by its high malignancy and correspondingly poor prognosis. It has an incidence of 3.19 in 100,000 people in the United States, affecting more males than females (1.6:1), with a late onset of disease at a median age of 64 years [76]. Typically, glioblastoma is mostly found within the white matter of the cerebral hemispheres with infiltration into the adjacent grey matter, especially in the frontal lobes [63], [77], [78]. However, glioblastoma can also be found throughout the central nervous system, for example in the brainstem, cerebellum [63], and spinal cord [79].

Gliomas located in the midline that are radiologically conspicuous for glioblastoma may also correspond to other diffuse gliomas, in particular diffuse midline glioma with H3-K27 alteration, which is a pediatric-type diffuse high-grade glioma [80]. Glioblastoma is the most aggressive and malignant neurocranial tumor type [81]. It often invades adjacent brain tissue very easily due to its infiltrative growth pattern [82] and may display a multifocal or multicentric [83] pattern, but very rarely metastasizes to other organs, although circulating tumor cells have been detected in blood samples from patients with glioblastoma [82], [84]. However, distant seeding via the cerebrospinal fluid (CSF) may be seen, for example, as so-called drop metastases in the spinal cord [85].

1.3.1.1. Etiology

Many risk factors have been investigated for their role in the development of glioblastoma, but only a few risk factors were found to be significantly correlated with the development of this tumor. In most cases, the etiology of this tumor type remains occult [86]. In very few cases, hereditary tumor syndromes such as Lynch syndrome, Li-Fraumeni syndrome, and Recklinghausen disease (neurofibromatosis 1) are associated with glioblastoma. Lynch syndrome describes the presence of an autosomal dominantly

inherited genetic disease due to a germline mutation in one of the DNA mismatch repair genes (e.g. *MSH2, MHL1, MSH6, PMS2*)[87]. The associated tumor syndrome is hereditary non-polyposis colon carcinoma (HNPCC) [88]. Li-Fraumeni syndrome is an autosomal dominant inheritance of a germline mutation in the tumor suppressor gene *TP53* [89]. Neurofibromatosis 1 is an autosomal dominantly inherited genetic disease, namely a neurocutaneous tumor syndrome caused by a mutation in the tumor suppressor gene *NF1* [90]. These tumor syndromes lead to the syndrome-specific occurrence of further tumor entities [91]. In addition, single nucleotide polymorphisms affecting various genes have also been associated with an increased risk of developing gliomas including glioblastomas [87].

Recognized external risk factors that may increase the risk of glioblastoma are non-ionizing irradiations of therapeutic indications, such as prophylactic cranial irradiation as a CNS prophylaxis in patients with acute leukemia [92] treated with a radiation dose of up to 24 Gray (Gy) [93]. In addition, survivors of nuclear accidents have been assigned an increased risk for developing various tumor entities, including gliomas [94], [95]. Other external influences on the development of glioblastoma that have been discussed are non-ionizing radiation from cell phones or of employment-related nature. Here, the data situation, however, is unclear and does not indicate a clearly increased risk [96], [97]. The presence of allergic or atopic diseases might be a protective factor that has been inversely correlated with the risk of glioblastoma [87].

1.3.1.2. Clinical Course

The physiological intracranial pressure of a supine, healthy, adult person is 7-15 mmHG [98]. If there is an increase in pressure in the neurocranium, the reserve volume, which corresponds to the inner and outer CSF space, is initially displaced and compressed [99]. A further increase leads to compression of the nerve tissue [100] and associated tissue, such as vascular structures, which ultimately leads to reduced perfusion of the nervous system itself [101]. The intracranial and intraspinal compartments are almost completely bony and are particularly at risk when there is an increase in volume/pressure intracranially [102]. The CNS in particular displays a high vulnerability to insufficient oxygen and energy supply [103]. High pressure or hypoxic microcirculation leads to

irreparable neurotoxicity [104]. In particular, the brainstem, which maintains and controls the vital vegetative system [105], is at risk of compression and hemorrhage caused by shearing of blood vessels. Serious increases in pressure lead to herniation of brain tissue into the incisura tentorii, secondarily into the foramen magnum, and thus to further fatal compression of the mesencephalon (also known as descending transtentorial/middle entrapment) or the brain stem (also known as descending tonsillar / lower entrapment). The mesencephalon and brainstem are sections of the CNS that both regulate vital functions and damage of these areas are acutely life-threatening [106].

Clinical presentations are according to the location of the glioblastoma and the consequent focal neurological deficits. Additionally, general symptoms of intracranial masses like headache, seizures, reduced conscience as well as increased brain pressure indications such as nausea, vomiting, meningism, and vegetative dysregulations are likely [107].

1.3.1.3. Diagnostics: Imaging

The diagnosis of glioblastoma is based on the CNS WHO classification, as mentioned above, so the tumor workup overall is multifaceted. As previously described, diagnosis is achieved by imaging, histology, and molecular pathology [72]. If a neurocranial neoplasia is suspected, appropriate imaging is usually initiated immediately [108], usually primarily in the form of a cranial computer tomography (cCT) with intravenous contrast administration [109]. Glioblastoma usually presents with characteristic radiomorphological features. In cCT evidence of a space-occupying lesion with irregularly configured margins, central cyst/necrosis, and ring-like enhancement after contrast media administration suggests the presence of a glioblastoma. Also notable is often the pronounced, associated medullary edema which presents itself as a perifocal hypodensity on cCT [110]. Often, this mass presents in a transboundary fashion, for example growing along the corpus callosum and presenting as a butterfly-like lesion affecting both frontal lobes, which is accordingly referred to as butterfly glioblastoma [111]. In cMRI, similar features as described above in cCT are considered indicative of glioblastoma [110]. In the different sequences, the tumor presents as mixed hyperintense in the T1 sequence and hyperintense in the T2 sequence [112], [113]. Although characteristic features are present in glioblastomas, diagnosis by imaging alone is not

sufficient. These radiologic features, particularly the marked perifocal edema and ring-like contrast affinity, are also seen in other space-occupying processes, such as intracerebral carcinoma or melanoma metastases, and brain abscesses [114]. Definitive clarification can only by achieved by tissue-based histological and molecular pathological investigations [115].

1.3.1.4. Diagnostics: Histopathology

Glioblastoma is characterized by its diffuse infiltration into neighboring brain tissue, mostly along white matter tracts but less frequently also involving the grey substance [116], [117]. Individual infiltrating cells may be found up to several centimeters from the macroscopically visible lesion and are therefore not seized by supramarginal resection [118]. Glioblastoma cells also appear as highly atypical and exhibit irregularly formed nuclei. Moreover, tumor cells may be arranged in a palisade-type manner around areas of necrosis [119], [120].

Also, there are frequent mitotic figures within the tumor, and proliferative activity assessed by determining the Ki-67 proliferation index is typically high [121]. The decisive feature is expansive or streak-like areas of necrosis and the formation of aberrant vessels in the tumor periphery and around necotic areas as a result of hypoxia. These malformed and glomeruloid-like vessels, caused by microvascular proliferation [122], are characterized by a particularly unfavorable luminal configuration that can trigger vascular thrombosis. In addition, the adjacent neoplastic tumor cells secrete pro-thrombotic factors that initiate the coagulation cascade, such as tissue factor (TF) [123], [124]. These consecutive microthrombi cause hypoxia, hypoglycemia, and an acidic micromillieu, which consequently lead to necrosis [125]–[129] A concomitant hemorrhagic component occurs most likely due to the formation of aberrant vessels, it is also likely that a tumoral infiltration of vessels might be the cause [130]. This association between microthrombosis and necrosis is more frequently observed in IDH-wildtype glioblastoma than in IDH-mutant CNS WHO grade 4 astrocytomas, which is probably due to the lower expression of TF and the lower occurrence of necrotic areas [123], [131]. In summary, these histopathological findings also translate into the radiomorphological appearance of glioblastoma, as previously described, e.g. the perifocal vasogenic edema for instance as

a consequence of e.g. vascular endothelial growth factor (VEGF) secretion by tumor cells and reactive as well as inflammatory processes [114]. The inflammatory infiltrates observed within glioblastoma are highly heterogeneous. In the vast majority of cases, up to 80% of the infiltrating immune cells are of myeloid, or more precisely of monocytic origins, such as macrophages and activated microglia. The macrophages located in the tumor tissue are also referred to as tumor-associated macrophages, the so-called (TAMs). Less predominant are lymphocytic cells, however, CD4+ T-helper cells, and CD8+ T-cells in particular have been found [132].

1.3.1.5. Diagnostics: Molecular pathology

Novel molecular pathologic markers of glioblastoma are considered in the latest CNS WHO classification (2021) [72]. In the previous CNS WHO classification (2016), a distinction was made between IDH-wildtype and IDH-mutant glioblastoma, the former typically presenting de novo, i.e. without an apparent lower-grade precursor tumor ("primary glioblastoma"), while the latter may develop from pre-existing lower grade IDH-mutant astrocytomas ("secondary glioblastoma") [133]. According to the most recent classification, only adult-type diffuse gliomas with an IDH-wildtype status are referred to as glioblastoma, while IDH-mutant astrocytic gliomas showing microvascular proliferation and/or necrosis are now referred to as astrocytoma, IDH-mutant CNS WHO grade 4. Other distinguishing molecular pathologic features include the histone H3 mutation status. This distinguishes IDH-wildtype glioblastoma from pediatric-type diffuse high-grade gliomas, including diffuse midline glioma, H3-K27-altered as well as diffuse hemispheric glioma, H3 G34-mutant [134], [135]. Furthermore, glioblastoma is characterized by at least one of the following histological or genetic alterations: necrosis, microvascular proliferation, *TERT* promoter mutation, *EGFR* gene amplification, a gain of chromosome 7 combined with loss of chromosome 10 (+7/-10). If one or more of these genetic alterations is fulfilled, the diagnosis of IDH-wildtype glioblastoma is considered, even if there is no evidence of necrosis or microvascular proliferation [136]–[138]. Other common chromosomal aberrations in IDH-wildtype glioblastoma include losses on 9p, in particular homozygous deletion of the cell cycle checkpoint inhibitor genes *CDKN2A* and *CDKN2B*. Other chromosomal losses are found on chromosome arms 13q, 22q, and

the gonosomes. In addition, trisomy of chromosomes 19 and 20 is often encountered [121], [139].

1.3.1.6. Therapy

As previously described, a full supramarginal resection of the tumor mass is only possible to a limited extent, since neoplastic cells have most likely already infiltrated into the adjacent brain tissue for distances of up to several cm from the tumor border [118]. Supportive approaches include fluorescence-assisted resection by application of 5-aminolevulinate (5-ALA), which accumulates strongly in the tumor cell and lights up under blue-violet light, thus allowing at least a generous macroscopic resection [140]. However, the therapy of glioblastoma is primarily a non-curative treatment, since recurrences arise from the tumor's microscopical seeding. Radiochemotherapy for at least 6 cycles followed by maintenance chemotherapy (according to Stupp,[141]) is recommended. Chemotherapy is preferably performed with temozolomide (TMZ), a cDNA alkylating agent [142]. Patients with a hypermethylated promoter of the DNA repair enzyme O6-methylguanine-DNA-methyltransferase (MGMT) in the tumor cells particularly benefit from this. Hypermethylation of the *MGMT* promoter suppresses the expression of the DNA repair gene, making the initiation of tumor cell death more likely, since repair of methylated DNA bases is no longer possible due to the inactivation of this gene. Patients with glioblastoma and a hypermethylated *MGMT* promoter thus benefit from the use of the alkylating chemotherapeutic agent temozolomide. This drug causes alkylation of the DNA purine bases, especially the guanine bases, so that a false pairing with thymine is initiated if not repaired by MGMT. Downstream of this, mismatch repair processes occur, leading to DNA double-strand breaks and thus to the initiation of apoptosis [143]–[145]. Since 2017, combined chemotherapy consisting of temozolomide and nitrosourea lomustine and concomitant radiotherapy has also been used for patients with an initial diagnosis of glioblastoma with hypermethylated *MGMT* promoter [146]. For patients with unmethylated *MGMT* status, targeted molecular therapy or trial participation should be considered [147]. Nonetheless, life expectancy after diagnosis is very short, with a median expectancy of 15 months with surgery and adjuvant radiochemotherapy and 3 to 6 months with surgical treatment alone [148], [149].

Therefore, new therapeutic options such as immunotherapy, tumor-treating fields, or targeted therapy are arising as possible new ways of tackling glioblastoma, however, each with still limited success [150].

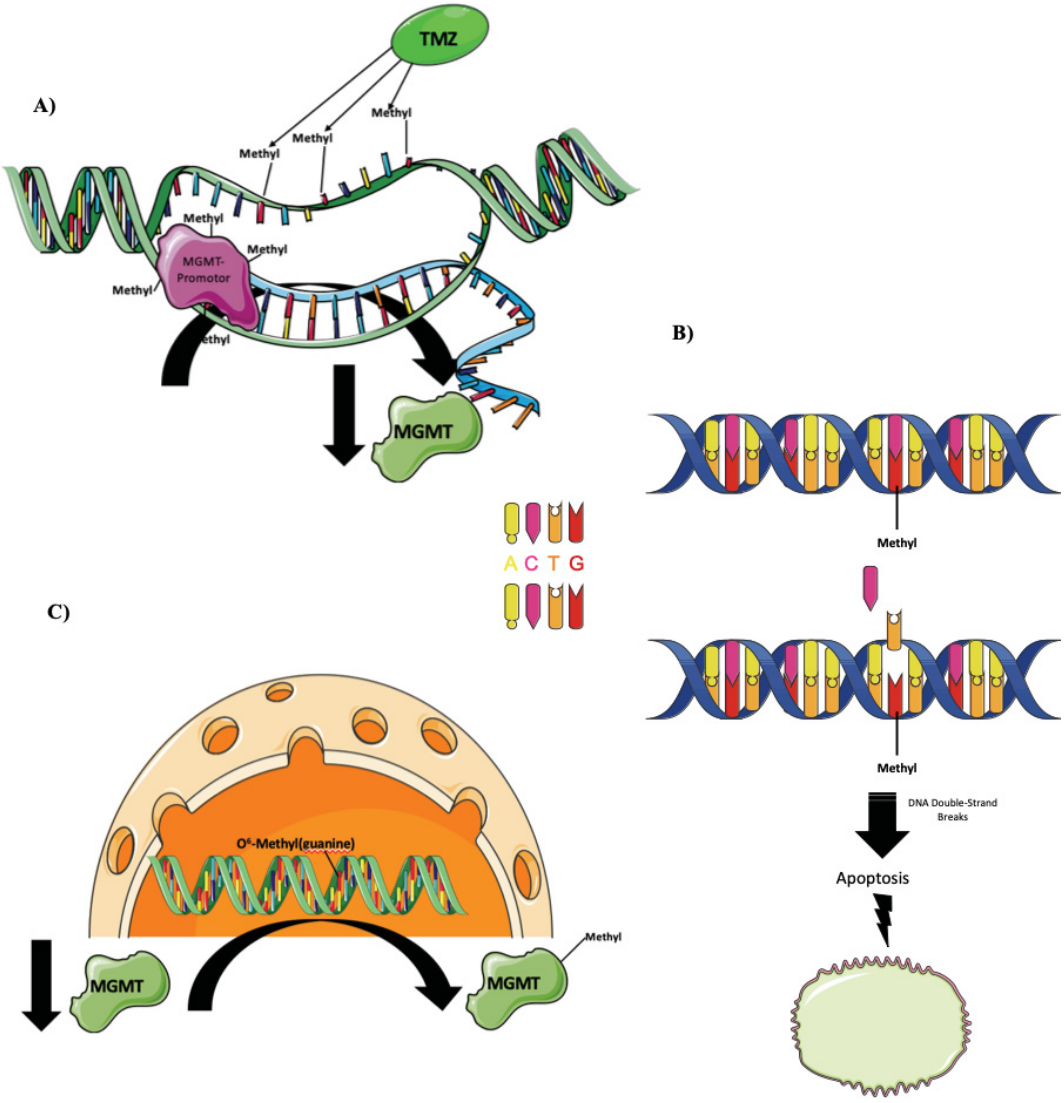

Fig.1: Molecular operating principle of action of TMZ and MGMT.
A decrease in MGMT expression due to hypermethylation of the *MGMT* promoter results in the inability to repair DNA guanine bases methylated by TMZ and mismatched. This is followed by the induction of mismatch repair processes, which cause DNA double-strand breaks and thereby induces apoptosis [143]–[145].*The image was designed independently using partial elements provided by Servier Medical Art, licensed under a Creative Commons Attribution 3.0 unported license.*

1.4. Cancer stemness

The “hallmarks of cancer” were postulated in 2000 by Douglas Hanahan and Robert Weinberg to describe the major cellular processes leading to tumorigenesis. The following causal hallmarks were defined: maintaining proliferative signals, avoiding growth suppressors, opposing cell death, enabling replicative immortality, generating angiogenesis, fostering chromosomal instability, and activating invasion and metastasis [151]. This was supplemented by two new hallmarks in the publication "Hallmarks of cancer next generation" by Hanahan and Weinberg (2011), namely aberrant metabolic pathways and immune system escape [152]. These proposals guided cancer science and revealed the complex and intertwined mechanisms of tumorigenesis [153]. In particular, a small population of cells within a tumor, namely cancer stem-like cells (CSCs) are known to express these acquired properties. Self-maintenance, self-renewal, and tumor progression are thus executed in a variety of ways [154]. CSCs are extremely resilient to unfavorable microenvironmental conditions, which at least in part is due to their dysregulated proliferative capacity and their evasion from external apoptosis signals [155]. Often, intratumoral heterogeneity of different cell types but also different degrees of differentiation is a common feature of malignant cancers, most likely due to the descent of a de-differentiated, stem-like cell type so that a divergent, asymmetric cell division into different cell types is possible [156]. It is also known that the influence of external factors can have a significant impact on the respective differentiation towards a certain cell type or cell-type-like state [157]. In this regard, too, influences of CSCs towards their microenvironment have been demonstrated. Consequently, CSCs condition their microenvironment and resident cells, for example, Mesenchymal Stem/Stromal Cells (MSCs) and immune cells attracted in the course of the normal immune response, to create a favorable environment in which tumor growth is enabled. The MSCs create a so-called tumor niche or tumor microenvironment that harbors and protects the CSCs as seen in the physiological stem cell niche [158]. In particular, CSCs interact with immune cells and promote the immigration of immunosuppressive immune cells. In particular, immune cells of myeloid origin are found in the immediate tumor environment. Numerous studies have demonstrated a negative correlation between cancer cell stemness and anti-tumor immunity. Accordingly, fewer cytotoxic immune cells and decreased activation of CD8+ and CD4+ positive T cells are found in the immediate tumor environment. Cancer

stemness positively correlates with the presence of regulatory T cells (Tregs), known for their immunosuppressive effect, tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells are found within the tumor niche [159]. For this reason, it is of considerable importance to understand the behavior of CSCs. Due to the asymmetric division of this cell type quiescent CSCs might remain despite curative therapy intention resulting in self-renewal and self-preservation of the tumor cells [160].

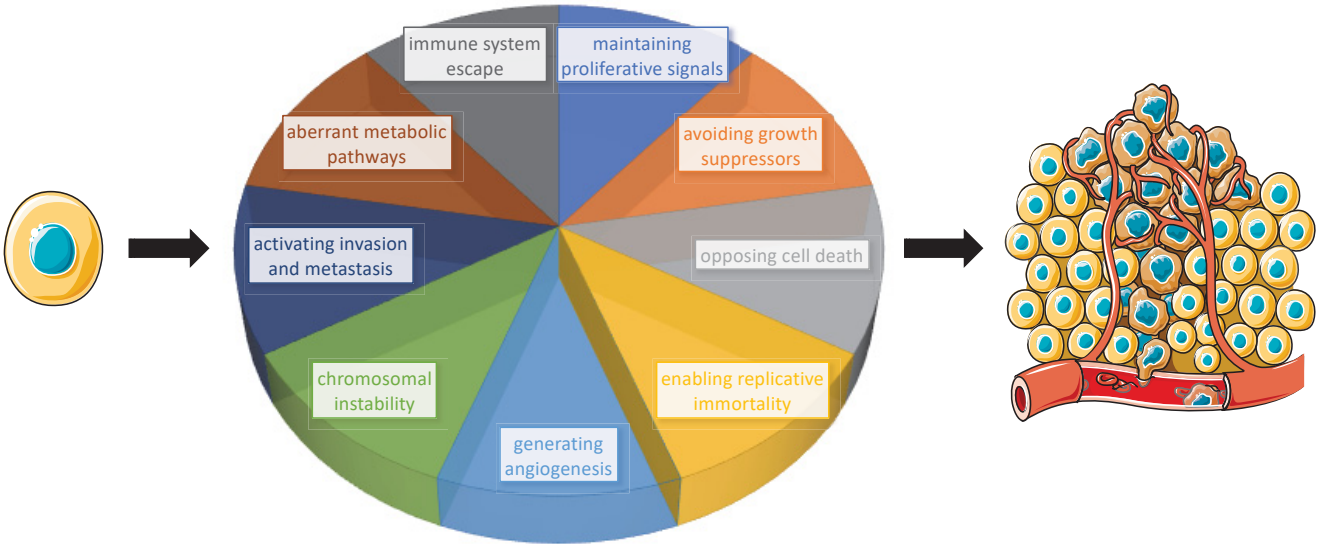

Fig.2.: Hallmarks of Cancer by Hanahan & Weinberg (2011).
The Hallmarks of Cancer according to Hanahan & Weinberg compromise the following characteristics of malignant transformation of cells: preservation of proliferative signals, avoidance of growth suppressors, opposition to cell death, facilitation of replicative immortality, induction of angiogenesis, chromosomal instability, and activation of invasion and metastasis, aberrant metabolic pathways and immune system escape. Modified according to D. Hanahan and R. A. Weinberg: "The hallmarks of cancer" & "Hallmarks of cancer: the next generation" [151]-[152]. The image was designed independently using partial elements provided by Servier Medical Art, licensed under a Creative Commons Attribution 3.0 unported license.

1.4.1. Glioblastoma stem-like cells

While there is a histologic similarity of glioblastoma cells to normal glial cells, this may not be indicative of the cell of origin. In recent years, substantial progress has been made in the understanding the origin of glioblastomas. In particular, the current understanding of CSCs and the intratumoral plasticity that they generate, meaning the ability to differentiate and dedifferentiate into different cell types and cell-type-like states complicates the assignment to one particular cell of origin. Accordingly, the identification of a causative cell lineage is difficult [45], [161]. Murine, *in vivo* experiments showed that a variety of CNS cell types can be transformed into malignant cells that share similar features as glioblastoma cells. The cell types elucidated are oligodendrocyte precursor cells (OPCs), neural precursor cells (NPCs), astrocytes, and neurons. The susceptibility to malignant transformation rises with an increasing degree of de-differentiation, as more stem cell-like properties are acquired. Genetic sequencing studies revealed evidence that NPCs residing in the subventricular zones may be relevant glioma progenitor cells [162]. The question is ultimately not resolved whether there is a causative progenitor cell or whether this is due to the dedifferentiation of different cell types [45]. Ultimately, intratumoral heterogeneity further complicates the assignment of a specific cell type as the precise cell of origin. Surface stem cell markers commonly used and discussed in the literature in this regard are CD133, CD44, and CD24. The corresponding data situation remains inconclusive probably due to the intratumoral heterogeneity of the presumably causative GSC. In complement to surface stem cell markers, special attention should be paid to stemness conferring transcription factors. In the context of GSC, significant transcription factors include SOX2, NANOG, and OCT4, which are expressed in particular in the context of embryogenesis of embryonic stem cells (ESC). In numerous studies, SOX2 has been identified as a transcription factor expressed by undifferentiated neural progenitor cells. Interruption of this SOX2-induced transcription resulted in low tumorigenicity of GSC. This also led to the assumption that GSCs originate from NPCs residing particularly in the subventricular zone. NANOG, on the one hand, maintains stem cell character via the modulation of the Hedgehog pathway and is maintained by CD24, among others. OCT4, as one of the oldest known stem cell markers, is also one of the best-studied and instrumental in conferring stem cell-like properties [163], [164].

In the context of glioblastoma, numerous stem cell signaling pathways have been discussed that mediate the formation and maintenance of GSCs, including Hedgehog, Notch, and last but not least the WNT-pathway [165]–[167].

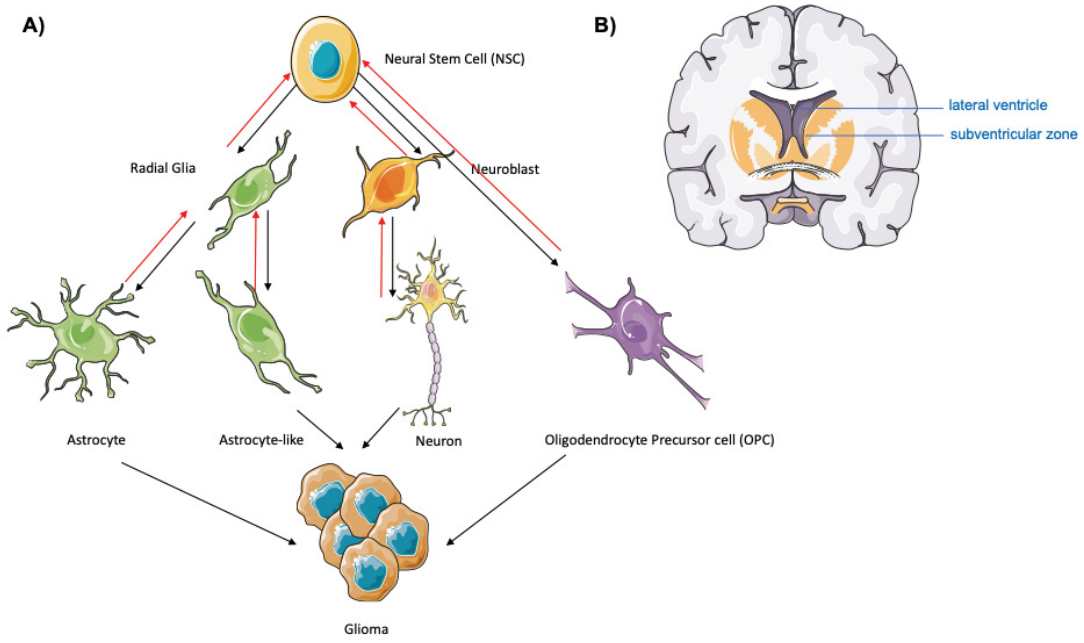

Fig.3: A-B) Suspected location & cells of Origin in Glioma.
The assignment of GSCs to a specific cell of origin is difficult due to their capacity to differentiate and dedifferentiate into several distinct cell types and cell-type-like states. As a result, it can be challenging to pinpoint a causal cell lineage [44], [159]. Murine *in vivo* investigations demonstrated that a range of CNS cell types can transform into glioblastoma-like malignant cells. The disclosed cell types include neurons, astrocytes, neural precursor cells (NPCs), and oligodendrocyte precursor cells (OPCs). As more stem cell-like characteristics are gained, the susceptibility to malignant degeneration increases with increasing degrees of de-differentiation. **B)** Research on genetic sequencing suggests that NPCs in the subventricular zones may be the most likely cells of origin for glioblastomas[160]. The image was designed independently using partial elements provided by Servier Medical Art designed according to [168], [169] , licensed under a Creative Commons Attribution 3.0 unported license.

1.5. WNT pathways

The WNT pathway is one of the most studied and phylogenetically highly conserved stem cell signaling pathways in vertebrates and invertebrates that mediates numerous essential processes of embryogenesis [12]. WNT is a portmanteau of the protein Wingless and Int-1. The term Wingless originates from the observation that mutations in the Wingless protein in *Drosophila melanogaster* lead to a lack of wing development. Int-1 activation (currently known as WNT1) experimentally led to the subsequent expression of breast cancer in murine organisms via genomic integration of murine mammary tumor virus (MMTV) [170]. Aberrant WNT activation has been detected in numerous tumors, including colorectal cancer, breast cancer, liver cancer, and other entities. This illuminates the importance of WNT in carcinogenesis [171].

WNT molecules undergo extensive post-translational modifications including being palmitoylated at a serine and cysteine residue in the endoplasmic reticulum via a membrane-bound O-acyl transferase called porcupine [172]–[174]. This palmitoyl group is essential for the adequate secretion of WNT molecules [173]. In addition, the WNT molecules are also glycolyzed at numerous sites [175]. The WNT molecules are transported by the transport protein Wntless [176]–[178]. A complex interplay of different protein superfamilies, such as the p24 and Baiser protein families, are involved in transporting the WNT molecule-laden transport proteins from the endoplasmic reticulum to the Golgi apparatus [179]–[181]. Anterogradely, Wntless and bound WNT molecules loaded into Golgi vesicles are transported to the cell surface and putatively released exocytotically. Resumption of the transport protein Wntless is presumptively clathrin-assisted [182]–[186]. The WNT molecules are thought to exert their effects primarily through autocrine or paracrine avenues [187]. Once secreted, they bind to the Frizzled receptors (Fz), a family of receptors that are G-protein coupled and accordingly span the cell membrane heptahelically [188], [189]. The WNT molecules bind to the cysteine-rich extracellular N-terminus. The receptor also interacts with so-called co-receptors such as lipoprotein-related receptors (LRP)-5/6, receptor tyrosine kinases, and ROR2 [188], [190]–[192]. Once a WNT molecule has bound appropriately to the Frizzled (Fz) receptor, an intracellular signaling cascade is triggered. First, Fz interacts with Dishevelled (Dsh). This is a downstream protein with at least three different protein

domains. This is of particular importance because the intracellular signal triggered by WNT can follow divergent signaling pathways depending on the interactions with the different Dsh domains [188], [193].

This divergence is reflected by three well-studied signaling pathways: the canonical WNT pathway, the noncanonical and planar cell polarity WNT pathway, and the noncanonical WNT/Ca^{2+} pathway. The canonical WNT pathway exerts its effect employing β-catenin, whereas the non-canonical pathways signal independently of β-catenin [172].

β-catenin is a cell adhesion protein and a transcription factor whose homeostasis is negatively affected by a complex of proteins that is composed of Axin2, glycogen synthase kinase 3 (GSK3), adenomatous polyposis coli protein (APC), protein phosphatase 2A (PP2A), and casein kinase 1α (CK1α). This complex phosphorylates the intracellular β-catenin when no WNT ligands bind to the Fz/ LRP5/6 receptor, which is thus consecutively phosphorylated. This in turn leads to ubiquitinylation of β-catenin, which is consequently degraded in the proteasome.

In the canonical pathway, binding of WNT ligands to Fz/LRP 5/6 leads to the accumulation of β-catenin in the cytosol while Axin2 is recruited to the cell membrane. Thus, the negative β-catenin regulator dissociates and β-catenin can accumulate in the cytosol and exert its effect intranuclearly together with other transcription factors that bind to the TCF/LEF promoter region [194]. In the nucleus, β-catenin initiates the transcription of numerous genes that can significantly promote oncogenesis in the course of aberrant activation, such as the protooncogene *c-Myc*, the cell cycle regulator *cyclin D*, the stem cell markers *CD44*, *Sox2*, *Nanog*, and *Oct 4*, the epithelial-mesenchymal-transition activators *ZEB1* and *Snail1,* the transporters *glucose-unilateral transporter 1 (GLUT1)*, *glucose-unilateral transporter 3 (GLUT3)* and *monocarboxylate transporter 1 (MCT-1), Axin2*, growth factors such as *fibroblast-growth-factor 20 (FGF20)* and *vascular endothelial growth factor (VEGF)*, just to name the most important β-catenin targets [194]–[207] The non-canonical WNT/ Ca^{2+} pathway is also initiated by the binding of WNT ligands to the Fz receptor, in which the corresponding Dsh domain is activated [193]. In contrast to the other WNT pathways, the Fz receptor interacts directly with a trimeric G protein and initiates the so-called Gq signaling pathway. This activates phospholipase C (PLC) or a cGMP-specific phosphodiesterase (PDE), which cleaves phosphatidylinositol 4,5-biphosphate into diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP3). The latter diffuses to the ER where it binds to a receptor and mediates calcium release from the ER into the cytosol. DAG can activate Cdc42 via protein kinase

C (PKC), which is instrumental in the so-called dorsoventral patterning of the neural tube. Furthermore, the release of calcium can also influence adhesion and migration. The non-canonical and planar cell-polarity pathway are initiated by WNT through binding to the Fz receptor, but instead of LRP 5/6 other co-activators are recruited that initiate a downstream signaling cascade involving morphogenesis and actin polymerization via the corresponding Dsh domain, such as NRH1, Ryk, PTK7 or ROR2 [12], [208], [209]. Many mechanisms of the WNT pathway have not been fully revealed to date, and it is believed that pleiotropy and complexity of the effects mediated by WNT extend well beyond what is known today [210]. The following chapters will focus on the effects mediated by WNT/β-catenin.

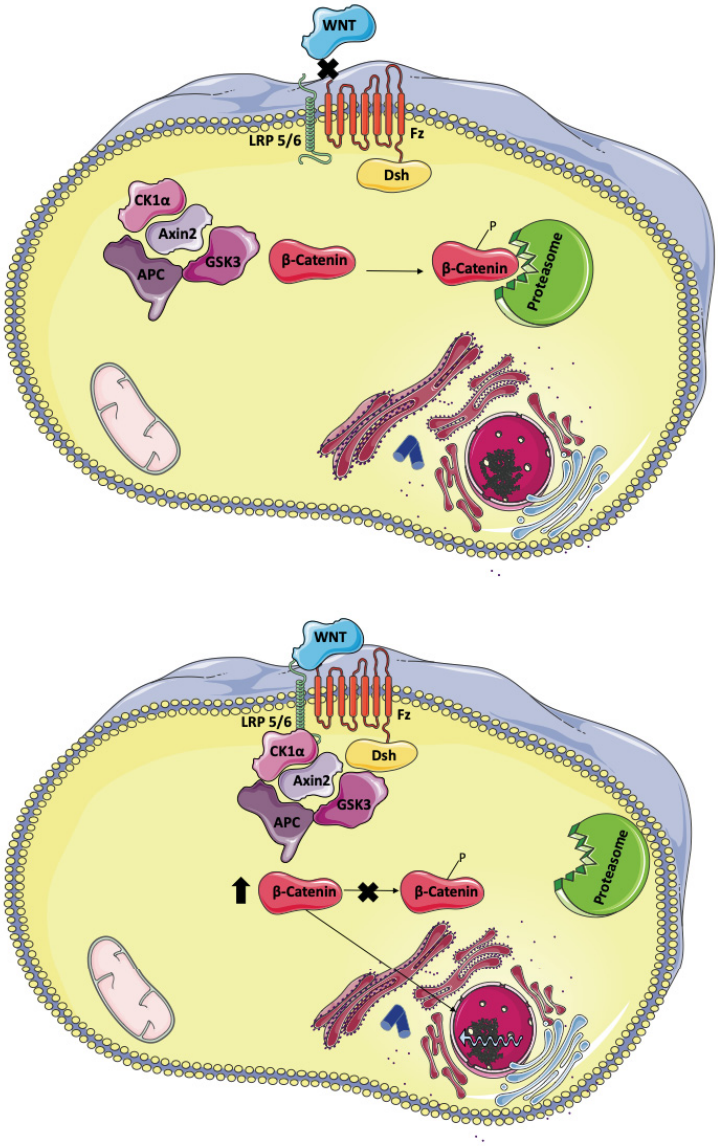

Fig.4: A-B) Canonical WNT-signaling in absence of WNT & in presence of WNT.
A combination of proteins adversely affects the homeostasis of the cell adhesion and transcription factor β-catenin: Adenomatous polyposis coli protein (APC), glycogen synthase kinase 3 (GSK3), protein phosphatase 2A (PP2A), and casein kinase 1 (CK1).When no WNT ligands bind to the Fz/LRP 5/6 receptor, the abovementioned proteins phosphorylate intracellular β-catenin. As a result, β-catenin is ubiquitinylated and then degraded in the proteasome. In the canonical pathway, binding of WNT ligands to Fz/LRP 5/6 results in the recruitment of Axin2 to the cell membrane, which results in the accumulation of β-catenin in the cytoplasm. Thus, nuclear translocation and regulation of gene transcription of β-catenin is enabled. The image was designed independently using partial elements provided by Servier Medical Art designed according to S. Shang, F. Hua, and Z. W. Hu, “The regulation of β-catenin activity and function in cancer: therapeutic opportunities” [192], licensed under a Creative Commons Attribution 3.0 unported license.

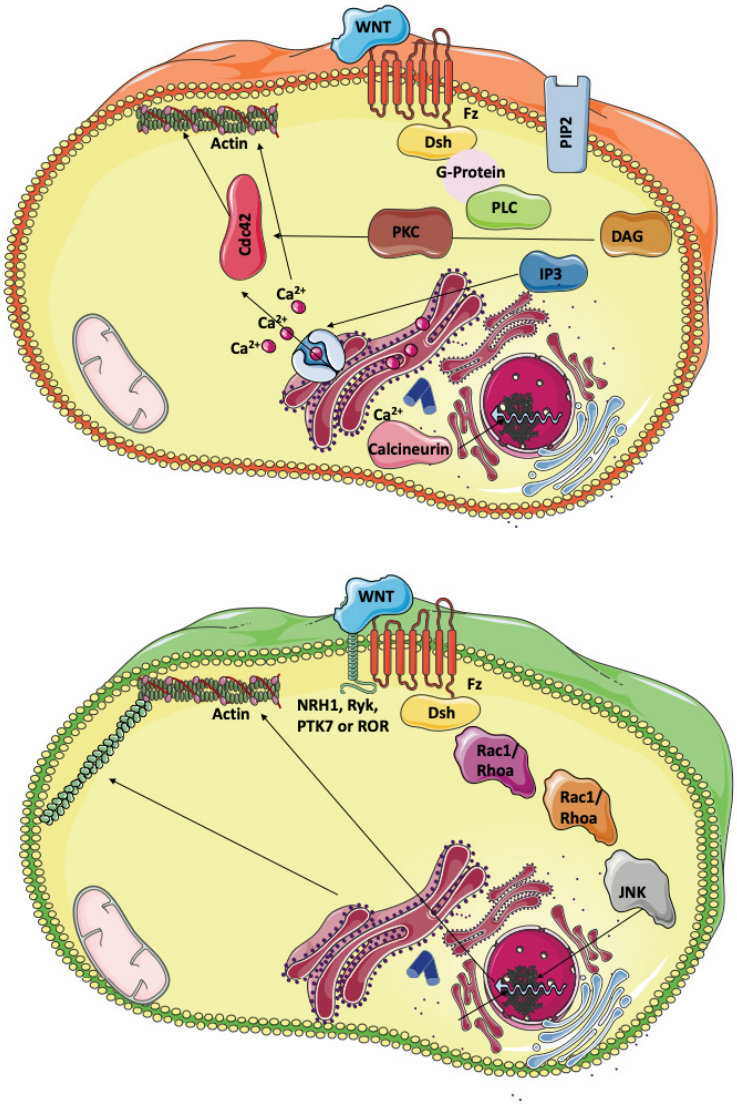

Fig.4: C-D) The non-canonical WNT/ Ca^{2+}/ pathway & The non-canonical WNT/planar cell polarity pathway in presence of WNT. The non-canonical Ca^{2}+/WNT pathway begins with the Fz-receptor initiating a Gq signaling cascade by interacting with a trimeric G protein. This triggers the phosphodiesterase (PDE) or phospholipase C (PLC), either of which cleaves phosphatidylinositol 4,5-biphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP3). IP3 diffuses to the ER where it interacts with a calcium receptor to mediate the release of calcium into the cytosol from the ER. The dorsoventral patterning of the neural tube is enabled by DAG mediated activation of Cdc42 via protein kinase C (PKC). Additionally, adhesion and migration are impacted by calcium release. In the WNT/planar cell polarity pathway, WNT triggers actin polymerization and the activation of co-activators other than LRP 5/6 (NRH1, Ryk, PTK7 or ROR2), including a cascade involving Rac1, Rhoa, and JNK. The image was designed independently using partial elements provided by Servier Medical Art designed according to I. Akoumianakis, M. Polkinghorne, and C. Antoniades, “Non-canonical WNT signalling in cardiovascular disease: mechanisms and therapeutic implications” [211] & [11], [206], [207].

1.5.1. WNT/β-catenin mediated epithelial-mesenchymal-transition

In particular, the influence of the aberrant WNT/β-catenin signaling pathway on the epithelial-mesenchymal transition in tumors has been extensively investigated in the past. At least three different mechanistic axes conditioning EMT can be distinguished: Loss of cell-cell adhesion, increased binding to extracellular matrix components(ECM), and enhancement of cell motility [212]–[217]. These axes are often of tremendous importance in ontogenesis and largely determine the dorsoventral patterning and accordingly the cell fates of migrated cells. This highlights that these are particular capabilities inherent to stem cell-like cells [218], [219]. Aberrations of stem cell signaling pathways lead to the acquisition of an invasive and mesenchymal-like phenotype in malignant cells in the adult organism and can trigger metastasis formation [220], [221]. In particular, the margins of a tumor and circulating tumor cells tend to express EMT markers and stemness markers. At the same time, these cells also frequently show a loss of expression of cell adhesion molecules(CAM) such as E-cadherins [218], [222]–[225]. β-catenin functions not only as a transcription factor as outlined in detail previously, but also as an intermediate cell adhesion protein and occurs in complex with E-cadherin and other catenins such as α- and γ-catenin. α-catenin links β-catenin to actin filaments of the cytoskeleton. When β-catenin dissociates from E-cadherin, for example through specific phosphorylation at tyrosine 654, it can diffuse into the cytosol, becomes transcriptionally active and enhances invasiveness. Thus, E-cadherin has a regulating function on the available β-catenin pool in the cytosol, as it directly binds β-catenin. Accordingly, when E-cadherin is depleted, the pool of β-catenin freely available in the cytosol is increased and thus nuclear translocation of β-catenin is enabled [226]. The WNT/β-catenin pathway itself maintains loss of E-cadherin through transcription of *ZEB1*, a β-catenin target gene and an epithelial-mesenchymal-transition transcription factor (EMT TF), that inhibits transcription of E-cadherin [227]. *SNAIL*, another EMT TF and target gene of β-catenin also directly represses transcription of E-cadherin and additionally stabilizes β-catenin [228], [229]. This corresponds to a self-amplifying mechanism for stem cell niche maintenance. The β-catenin protein also regulates the transcription of the stem cell marker CD44 [201]. The latter binds to the extracellular matrix and also enhances WNT/β-catenin signaling via phosphorylation of LRP6 [201], [230], [231].

β-catenin also induces the expression of L1CAM1 (L1), a neural immunoglobulin-like adhesion protein, this also suppresses the expression of E-cadherins and positively regulates β-catenin [212], [232], [233]. The processes described above describe a very complex and intertwined interplay between EMT and Stemness which maintains and enhances itself. EMT-positive miRNAs of the WNT/β-catenin pathway are currently under investigation and may provide novel therapeutic targets against metastatic disease [234].

1.5.2. WNT/β-Catenin and its implication in cancer metabolism

1.5.2.1. WNT/β-Catenin and its implication in cancer glucose metabolism

Aberrant WNT/β-catenin activation in tumor cells mediates not only stem cell-like properties and the associated capacity for self-maintenance, self-renewal, and the ability to undergo epithelial-mesenchymal transition, but also drives metabolic reprogramming, which has been included in the most recent (2011) version of the Hanahan and Weinberg hallmarks of cancer [152]. This acquired ability of metabolic adaptability and plasticity of tumor cells leads to the resilience of stem cell-like cells and thus causes unique challenges in therapy. This resilience confers resistance to therapy and underscores the adaptability of these cells to extreme microenvironments [210], [235]. In particular, the WNT/β-catenin pathway confers pleiotropic metabolic capabilities that maintain the self-preservation of neoplastic cells. The following paragraphs briefly review the effects mediated by WNT/β-catenin on glycolysis, glutaminolysis, and lipogenesis [235]. In the glucose metabolism of tumor cells in general, it is important to emphasize the widely known fact that neoplastic cells exhibit the so-called Warburg effect, which is also called aerobic glycolysis [236]. In comparison to non-neoplastic cells, neoplastic cells do not cover their energy requirements by mitochondrial oxidative phosphorylation under normoxic conditions. At first glance, this would seem logical, since the ATP yield per molecule of glucose via oxidative phosphorylation is significantly higher than that of (anaerobic) glycolysis, by which 32 ATP are obtained per molecule of glucose. In

anaerobic glycolysis, which takes place under physiological conditions and is energetically more inefficient, only 2 ATP are obtained per molecule of glucose [237]–[239]. This raises the question of why tumor cells prefer aerobic and more energy-inefficient glycolysis. This is probably based on the fact that cellular energy production is decoupled from mitochondrial function, since mitochondrial dysfunction is often present in neoplastic cells. In addition, a number of other advantages of aerobic glycolysis of tumor cells emerged in numerous studies. Increased aerobic glycolysis not only produces more pyruvate but also lactate [237], which leads to an acidification of the pH, which in turn promotes the hostile tumor microenvironment (TME) and causes the acquisition of a migratory and invasive phenotype and the secretion of VEGF [240], [241]. WNT promotes aerobic glycolysis in tumor cells through numerous processes. First, it increases glucose influx in tumor cells through transcription of the unilateral glucose transporters GLUT1 and GLUT3. This makes sense since the ATP yield per molecule of glucose is significantly lower by aerobic glycolysis than by oxidative phosphorylation. Accordingly, glucose influx must increase to meet the energy requirements of the rapidly proliferating cells. *GLUT1* and *GLUT 3* are also target genes of the oncoprotein c-Myc, which is itself a direct β-catenin target [242], [243]. C-Myc also induces the expression of the glycolysis key enzyme phosphofructokinase (PFK) [243], [244]. The glycolytic phenotype is further enhanced by suppression of mitochondrial function mediated by the WNT ligand WNT5b [245]. In addition, pyruvate carboxylase is induced by the WNT/β-catenin signaling pathway, which carboxylates pyruvate to oxaloacetate, a glucogenic amino acid, and leads to gluconeogenesis, i.e. the opposite effect of glycolysis, or removes pyruvate from the subsequent steps to oxidative phosphorylation [246], [247]. In addition, WNT increases the expression of pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates pyruvate dehydrogenase and thus inactivates it, thus preventing the conversion of pyruvate into acetyl-CoA. The benefit of this bypass is essentially that by bypassing oxidative phosphorylation, among other things, the generation of reactive oxygen species (ROS) is bypassed, which negatively influence the development of a stem cell-like phenotype and thus interferes with the establishment of a stem cell niche [247], [248]. *Hypoxia-inducible factor 1-alpha (HIF1α)* is a target gene of WNT/β-catenin even in normoxia and its gene product also reduces mitochondrial biogenesis via MXI2, a c-Myc antagonist, and also modulates cytochrome C oxidase, a component of the mitochondrial respiratory chain [247]–[251]. In addition, it also activates pyruvate dehydrogenase kinase and reduces ROS formation

via the same mechanism mentioned above by preventing oxidative phosphorylation. In addition, cell death caused by oxidative stress is prevented, and a stem-cell-like phenotype maintained [248], [252], [253]. Oxidative cell stress is also triggered, for example, by irradiation/chemotherapy - neoplastic cells obtain therapeutic resilience by avoiding the generation of ROS altogether, thus posing a challenge in developing efficient therapy strategies [254].

1.5.2.2. WNT/β-Catenin and its implication in cancer amino acid metabolism

As previously described, aerobic glycolysis is of immense importance for the metabolism of neoplastic cells. Substrates for this are also obtained via the intermediate metabolites of the TCA, such as amino acid derivatives like alpha-ketoglutarates, malates, and pyruvates [235], [237], [255]. It should be emphasized that the various metabolic pathways are closely interlinked and can merge into one another, so special attention must be paid to the amino acid metabolism of tumor cells [235]. In particular, glutaminolysis in normal and especially in malignant cells is of special importance in cells of the central nervous system. In the literature, tumor cells are also referred to as glutamine-addicted, since numerous metabolic pathways can be triggered via this glucogenic amino acid. An increased pool of glutamine has also been previously linked to increased proliferation, viability, and migration in tumors [256], [257]. The WNT/β-catenin signaling pathway positively influences glutamine influx into the cell, on the one hand via the induction of c-Myc, which encodes numerous genes of glutamine metabolism, such as glutaminase *SLC1A5*, a sodium-dependent transporter for L-glutamates, D-aspartates, L-aspartates, and L-cysteines. In neurons, in particular, this also serves to remove the excitatory L-glutamate from the synaptic cleft and ensures the influx of L-glutamates and L-cysteines in particular, and thus the production of gluthatione in order to neutralize reactive oxygen species [258]–[262]. The amount of glutamate formed is also dependent on the stem cell marker and β-catenin target protein CD44 [201], since the amount of CD44 molecules expressed correlates positively with the expression of SLC1A1 and thus with a positive influx of L-glutamate and L-cysteine [263]. Furthermore, an inverse correlation was

found between a decreasing intracellular glutamine amount and increased phosphorylation of β-catenin. Accordingly, a glutaminergic phenotype can be assumed in cells upregulating WNT/β-catenin. Glutamate and ammonia are obtained via glutaminolysis, but ammonia is mostly secreted. The glutamate is deaminated to alpha-ketoglutarate in the mitochondria, thereby releasing another ammonia group. Alpha-ketoglutarate is one of the most important anaplerotic substrates for the TCA in neoplastic cells [264], [265]. Glutaminolysis ultimately proceeds to reduce glutamine to lactate, $NADPH/H^+$ is recovered for biosynthetic processes such as fatty acid synthesis, and numerous anaplerotic substrates are recovered for the TCA, such as the precursor malate [257]. Reduction equivalents such as $NADH/H^+$ and flavin adenine dinucleotide (FADH2) are obtained via TCA metabolism and non-essential amino acids (NEAA) are consequently regenerated. These are then used for gluconeogenesis and ketogenesis [266], [267]. In all these glutaminolytic processes, the nitrogen-containing ammonia groups are mostly secreted, suggesting that glutamine not only serves as a nitrogen source but also serves as a major carbon source contributing to macromolecule synthesis, gluconeogenesis and replenishing various biosynthetic pathways [22], [268].

1.5.2.3. WNT/β-Catenin and its implication in cancer lipid metabolism

Lipid metabolism in tumor cells is of considerable importance for tumor metabolism. As previously described, the different metabolic pathways interfere with each other. If necessary, ketogenic amino acids obtained by the TCA are used for ketogenesis or fatty acid biosynthesis. Lipids are not only essential energy sources but also important for the integrity of the cell membrane. The amount of lipids produced correlates with tumorigenicity or tumor progression [235]. β-catenin knockdown in breast cancer cells revealed that overall fewer lipogenic enzymes such as the citrate carrier, acetyl-CoA carboxylase, and fatty acid synthase, Mlxipl, and SREBf1 were expressed. β-catenin is also significantly involved in lipid desaturation. This is mediated by increased expression of sterol regulatory element-binding protein 1 (SREBP1-1) [269], [270]. WNT/β-catenin promotes fatty acid oxidation and stabilization of β-catenin thereby. These unsaturated fatty acids were reported to in turn inhibit β-catenin degradation [270], [271].

1.5.3. Therapies targeting WNT pathway

Aberrant WNT/β-catenin activation has been linked to increased tumorigenicity in numerous studies and has also been shown to be oncogenic in glioblastoma, in addition to numerous other aberrant signaling pathways [235]. Accordingly, the development of therapeutics that inhibit this pathway is an obvious therapeutic approach. The complexity of the previously described WNT/β-catenin signaling pathway allows pharmacological intercalation at different regulatory sites [272].

There are various porcupine (PORCN) inhibitors such as LGK974, which was also experimentally investigated in the publication on which this dissertation is based, and other PORCN inhibitors such as ETC-1922159 and CGX1321. These inhibitors inhibit the endoplasmic reticulum protein porcupine, which palmitoylates WNT and thus enables the secretion of WNT molecules for further transport into the Golgi apparatus. Thus, WNT signaling is inhibited at its root [272]–[274]. These PORCN inhibitors have been tested with preclinical success, showing inhibited bone metabolism as a side effect. It has been previously shown experimentally that WNT is essential for a regulated and physiological bone metabolism [275].

Another approach is the inhibition of the frizzled receptors. This is made feasible by the secreted frizzled-related proteins (SFRP) and the secreted frizzled-related proteins peptides (SFRP peptides). These bind to the Fz receptors and compete for these binding sites with the WNT proteins. Preclinically, a tumor-suppressing effect has been observed [276]–[279]. In some studies, there is an additional WNT/β-catenin inhibitory pharmacological group, the Fz antagonists/monoclonal antibodies such as vantictumab, which binds to Fz 1,2,5,& 7 and inhibits the WNT signaling pathways that are transduced into the cell [280]. In clinical studies, this also caused nausea, diarrhea, and other abdominal complaints as adverse side effects. Then, there is ipafricept, which is a recombinant fusion protein that binds to Fz 8 and thus inhibits the propagated WNT signaling [281], [282]. Beyond that, there is the pharmaceutical compound OTSA101DTPA-90Y, which is labeled with a β-emitting yttrium atom and binds to Fz 10 [283]. Vantictumab and Ipafricept, in addition to their WNT/β-catenin suppressing effect, also show disturbances in bone metabolism [280], [281].

Another pharmacological approach is LRP degradation or Fz endocytosis. Some drugs intervene here, such as salinomycin, rottlerin, and monensin, which phosphorylate LRP6 and thus mediate its degradation. Niclosamide induce the endocytosis of Fz 1, which leads to β-catenin destabilization induced by WNT3A. The associated side effects are rather unspecific such as rash, taste disturbances, and nausea [284]–[288]. Another approach is based on the inhibition of tankyrase, an enzyme that destabilizes Axin1and thus prevents the formation of the β-catenin degradation complex, inhibition of tankyrase would promote the degradation of β-catenin via Axin1, GSK3b APC, and CK1α. A positive effect was shown particularly in neoplastic cells that exhibited a mutation of *APC*. Pharmaceuticals that were clinically tested for this purpose were: XAV939, JW-55, RK-287107, and G007-LK [289]–[294]. CK1 agonists were also evaluated, pyrvinum is an approved antihelminthic drug that promotes the activity of CK1 and thus positively affects the β-catenin degradation complex [295].

β-catenin-mediated transcription can also be modulated pharmacologically [272]. For example, PRI-724 can negatively affect the interaction of cAMP responsive element binding protein (CREB) binding protein (CBP) and β-catenin, and thus the resulting β-catenin-induced transcription [296]. Berberine is a phytotherapeutic agent derived from barberry, which also affects WNT/β-catenin-induced gene transcription, some reports suggest a possible use in hypertensive treatment, but the evidence remains unclear [297], [298].

β-catenin also forms complexes with transcriptional coactivators such as BCL and PYGO and thus exerts its transcriptional effects [299], [300]. Carnosic acid, compound 22, and SAH-BLC9 prevent the formation of these complexes. In addition, the CK1 agonist pyrvinium promotes the degradation of the coactivator PYGO [295], [301], [302]. Besides, specific WNT/β-catenin target genes can be inhibited by sm0852, which inhibits the serine and arginine-rich splicing factor (SRSF) by phosphorylating it, thus preventing its spliceosome activity and the resulting post-transcriptional modification of the β-catenin transcript [303]. In addition, there is also methyl 3-[(4-methylphenyl)sulfonyl]amino-benzoate (MSAB) which is a compound that binds β-catenin directly and thus mediates the proteasomal degradation of β-catenin. Since β-catenin initiates essential and pleiotropic processes that are also physiologically necessary, the associated adverse side effects are rather nonspecific, such as fatigue, abdominal discomfort, and thrombocytopenia [301], [304].

1.6. Clinical implications of glucose metabolism in cancer

A positive correlation between hyperglycemia and poor clinical outcome of cancer has been observed in numerous clinical studies [305]. Hyperglycemia in glioma patients was associated with lower survival, increased recurrence, increased tumorigenicity, and disease progression [306]–[308]. For example, hyperglycemia may be caused by a metabolic disease such as diabetes mellitus or may be a therapy-associated adverse effect, such as the use of dexamethasone, a very potent glucocorticoid, which is frequently used in glioblastoma patients for the treatment of the associated brain edema. Hyperglycemia is accompanied by hyperinsulinemia, whether therapeutically induced in diabetic patients or physiologically induced [305]. Insulin not only mediates the uptake of glucose into the rapidly proliferating and starving tumor cells but also shows a structural similarity to insulin-like growth factor 1 (IGF-1), which is secreted by tumor-associated macrophages and positively influences the tumor microenvironment and the tumor cells for proliferation [309]–[311]. In addition, hyperglycemia positively affects the energy balance of tumor cells, allowing for survival and growth of the tumor mass [312]. The undesirable side effects associated with hyperglycemia, such as the predisposition to (wound) infections, sepsis, and susceptibility to infections in general is thought to be due to the reduced endogenous immune defense [305], [313], and thus an impaired immune answer against the tumor as a consequence. Hyperglycemia through corticosteroids bears additional risks such as corticosteroid-induced myopathy and adrenal impairment [314]. However, improved clinical outcome was also observed in glioblastoma patients with type 2 diabetes mellitus who had previously received metformin medication. However, experimental studies showed metformin-induced inhibition of glioblastoma proliferation and migration, and thus an inverse correlation between hyperglycemia and prior metformin therapy and clinical outcome [315].

2. Objectives of this thesis

In recent years, considerable progress has been made to understand the tumorigenesis, self-renewal, and self-preservation of glioblastoma and developing appropriate therapeutic strategies. The malignant growth and therapy resistance of IDH-wildtype glioblastoma is thought to be driven by a subpopulation of tumor cells with cancer stem-like cell (CSC) properties [316]. Employing a high-throughput *in vitro* drug screen, LGK974 and berberine were identified as two of twelve drugs that impair wingless (WNT) signaling, thereby sensitizing glioblastoma stem-like cells (GSCs) to glucose starvation-induced cell death [297], [317]. In recent studies, special attention has been paid to glioblastoma stem cells (GSC) and the corresponding stem cell signaling pathways [318]. In particular, the WNT pathway has been and continues to be thoroughly investigated as a phylogenetically highly conserved stemness pathway [319]. However, the extent to which metabolic implications of the WNT pathway exist and how these affect tumor growth and survival is poorly understood [15]. There is a large body of work that has elucidated the role of the WNT pathway in beta-oxidation and glucose metabolism of various tissues and tumor entities, but few to no analyses have been done on other metabolites [235]. Using gas chromatography-mass spectrometry, GSCs were investigated under pharmacological WNT inhibition by LGK974 in this study and examined for the intracellular concentrations of a selection of amino acids, oncometabolites, tricarboxylic acid cycle metabolites, and a selection of lipophilic metabolites. This work should essentially address WNT/β-catenin mediated resistance under glucose deprivation of GSC and illuminate how metabolic stress in the form of glucose deprivation affects the viability, clonogenicity, and invasive capabilities of glioblastoma stem cells. In addition, it should be studied whether additional pharmacological inhibition of the WNT pathway significantly reduces survival in nutrient-limited environments and which impact this approach might have on the intracellular metabolic composition of GSCs. Correlatively, the transcriptome of GSCs under pharmacological WNT inhibition and nutrient restriction (glucose depletion) should be investigated in order to identify β-catenin dependent and independent genes, as well as to determine genetic alterations due to nutrient limitation. The data summarized in this publication-based thesis have been published as an original paper entitled

"WNT/β-catenin-mediated resistance to glucose deprivation in glioblastoma stem-like cells"[320]. This paper is reprinted in this dissertation on the following pages.

3. Results

3.1. Publication

Suad Yusuf, Philippe Aretz, Ann-Christin Nickel, Philipp Westhoff, Amit Sharma, Nan Qin, Marc Remke, Hans- Jakob Steiger, Daniel Hänggi, Hongjia Liu, Hongde Liu, Guido Reifenberger, Jarek Maciaczyk. WNT/β-Catenin-Mediated Resistance to Glucose Deprivation in Glioblastoma Stem-like Cells. Cancers 14, Issue 13, 3165, 2022 [1].

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WNT/β-Catenin-Mediated Resistance to Glucose Deprivation in Glioblastoma Stem-like Cells

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Simple Summary: The malignant growth and therapy resistance of isocitrate dehydrogenase (IDH)-wildtype glioblastoma is thought to be driven by a subpopulation of tumor cells with cancer stem-like cell (CSC) properties. Employing a high-throughput in vitro drug screen, we identified LGK974 and berberine as drugs that impair wingless (WNT) signaling and can thereby sensitize glioblastoma stem-like cells (GSCs) to glucose starvation-induced cell death. The main goal of this study was to characterize the role of the WNT pathway in mediating the survival and metabolic plasticity of GSCs under nutrient-restricted growth conditions. Gas chromatography mass spectrometry (GC-MS) was used to determine WNT-specific alterations of intracellular metabolites in GSCs grown under nutrient restriction, i.e., glucose depletion, or under standard conditions. Metabolic fingerprints hold the promise to complement classic biomarkers, thus potentially aiding the prediction of tumor behavior and patient prognosis.

Abstract: Isocitrate dehydrogenase (IDH)-wildtype glioblastoma is the most common primary malignant brain tumor. It is associated with a particularly poor prognosis, as reflected by an overall median survival of only 15 months in patients who undergo a supramarginal surgical reduction of the tumor mass followed by combined chemoradiotherapy. The highly malignant nature of IDH-wildtype glioblastoma is thought to be driven by glioblastoma stem-like cells (GSCs) that harbor the ability of self-renewal, survival, and adaptability to challenging environmental conditions. The wingless (WNT) signaling pathway is a phylogenetically highly conserved stemness pathway, which promotes metabolic plasticity and adaptation to a nutrient-limited tumor microenvironment. To unravel the reciprocal regulation of the WNT pathway and the nutrient-limited microenvironment, glioblastoma cancer stem-like cells were cultured in a medium with either standard or reduced glucose concentrations for various time points (24, 48, and 72 h). Glucose depletion reduced cell viability and facilitated the survival of a small population of starvation-resistant tumor cells. The surviving cells

demonstrated increased clonogenic and invasive properties as well as enhanced chemosensitivity to pharmacological inhibitors of the WNT pathway (LGK974, berberine). Glucose depletion partially led to the upregulation of WNT target genes such as *CTNNB1*, *ZEB1*, and *AXIN2* at the mRNA and corresponding protein levels. LGK974 treatment alone or in combination with glucose depletion also altered the metabolite concentration in intracellular compartments, suggesting WNT-mediated metabolic regulation. Taken together, our findings suggest that WNT-mediated metabolic plasticity modulates the survival of GSCs under nutrient-restricted environmental conditions.

Keywords: glioblastoma; cancer stem-like cells; glucose starvation; cancer metabolism; WNT/β-catenin

1. Introduction

Gliomas are the most common primary malignant brain tumors [1]. They are classified into distinct tumor types based on the combination of histopathological features and molecular biomarkers according to the World Health Organization (WHO) classification of central nervous system (CNS) tumors [2]. Among the diffusely infiltrating gliomas in adults, the WHO classification distinguishes three major tumor types, namely the IDH-mutant astrocytomas of CNS WHO grades 2, 3, or 4, the IDH-mutant and 1p/19q-codeleted oligodendrogliomas of CNS WHO grades 2 or 3, as well as the IDH-wildtype glioblastomas of CNS WHO grade 4. Malignant glioma growth and resistance to therapy are thought to be driven by a subpopulation of tumor cells with stem-like features, including the ability of self-renewal and an adaptability to extreme and adverse microenvironmental conditions in terms of nutrient availability, especially during rapid tumor growth [3–6]. Tumor expansion leads to hypovascularized, hypoxic, and nutrient-deprived tumor areas consequently favoring the survival of subpopulations of nutrient stress-resilient cells [7]. The study of the metabolic plasticity and reprogramming of tumor cells in nutrient-limited microenvironments in IDH-wildtype glioblastoma is a promising area of research that may delineate mechanisms promoting tumor cell survival [8]. Stress resilience has been attributed to stem-like tumor cells in glioblastomas, with stemness playing an essential role in promoting the resilience, self-renewal, and metabolic adaptability of glioblastoma cells [9]. Various stemness factors and phylogenetically conserved stemness pathways, such as the WNT pathway, have been implicated as drivers of cancer cell stemness and tumor growth in different types of cancers, especially breast cancer, colorectal cancer, prostate cancer, and glioblastoma [10]. WNT signaling has pleiotropic effects on cell physiology and development during embryogenesis and organogenesis, especially in nervous tissue, and in maintaining metabolic homeostasis. WNT signaling can be divided into several independent pathways such as the WNT/β-catenin-dependent (referred to as the canonical WNT pathway) and the non-canonical pathway. The non-canonical pathway is further subdivided into the planar cell polarity and the WNT/Ca^{2+} pathway, each inducing different downstream cascades and thereby promoting different cellular effects. The most studied WNT pathway is the canonical WNT signaling pathway, which regulates the abundancy of the transcriptional co-activator β-catenin that controls key developmental processes [11]. Altered β-catenin signaling, especially via the aberrant activation of the canonical WNT pathway, plays an important role in promoting tumor growth in various tumor types, including glioblastoma [12]. Recently, our group has shown that β-catenin, together with CCL2, promotes monocyte migration towards glioblastoma cells [13]. In particular, WNT-mediated β-catenin signaling is thought to promote metabolic changes in cancer [14]. For example, the transcriptional upregulation of monocarboxylate-transporter 1 (MCT-1) has been linked to β-catenin-mediated promoter activation via binding to *TCF/LEF* sites, which consequently leads to lactate secretion and increased aerobic glycolysis [15]. It has been discussed that activation of the canonical WNT pathway may lead to the activation of aerobic glycolysis through the upregulation of pyruvate

dehydrogenase (PDH), pyruvate kinase M2 (PKM2), lactate dehydrogenase A (LDH-A), a sodium-dependent unspecific amino acids transporter (SLC1A5), and glucose transporter 1 (GLUT1). Non-canonical WNT signaling also enhances glycolysis by activating the Akt-mTOR pathway [14]. Fatty acid oxidative metabolism is also enhanced by mTORC1 and β-catenin signaling [16]. In addition, glucose-6-phosphate dehydrogenase, increased by mTOR as a consequence of an upregulated pentose phosphate bypass, leads to the enhanced availability of reduction-equivalents, such as co-enzyme nicotinamide adenine dinucleotide phosphate ($NADPH/H^+$), and provides phosphoribosyl pyrophosphate (PRPP) for purine and pyrimidine synthesis, thus supplying more substrates for efficient DNA synthesis [17].

In the present study, we focused on cellular models of IDH-wildtype GSCs and investigated the impact of glucose concentration on the cellular metabolism and biological features of GSCs in vitro. As a proof of principle, we assessed the effects of glucose deprivation on the WNT/β-catenin pathway in glioblastoma and evaluated the consequences of pharmacological WNT inhibition on GSCs under glucose-restricted conditions.

2. Materials and Methods

2.1. Cell Culture and Glucose Starvation

We used three GSCs models in our study: GBM1 cells (generously provided by A. Vescovi, San Raffaele Hospital, Milano, Italy), JHH520 cells (generously provided by G. Riggins, Johns Hopkins, Baltimore, MD, USA), and BTSC233 cells (generously provided by M.S. Carro, Freiburg University, Freiburg im Breisgau, Germany). As a standard protocol for glioblastoma stem-like cancer cell enrichment, we cultured these cell lines in Neurobasal™-A medium without D-glucose and sodium pyruvate, and substituted glucose equivalent to the standard cell culture glucose concentration (450 mg/dL) using a glucose solution (200 g/L; Gibco BRL). The starvation protocol was conducted as follows: 5 mL of cells cultured in standard cell culture glucose concentration (450 mg/dL) were collected and centrifuged for 5 min (min) at 1000 rpm. The supernatant was then removed and the harvested cells were resuspended in Neurobasal™-A complete medium without D-glucose for a defined period of time. In addition, 2% B27 supplement (Gibco), 20 ng/mL human bFGF (Peprotech, Rocky Hill, NJ, USA), 20 ng/mL human EGF (Peprotech), 5 µg/mL heparin (Sigma, Merck KGaA, Darmstadt, Germany), and 1% penicillin–streptomycin–fungicide mixture (Gibco) were added to the medium. The cell lines were cultured at 37 °C and 5% CO_2.

2.2. Cell Viability

To evaluate cell viability, 5×10^3 cells of each glioblastoma cell line were resuspended in 100 µL Neurosphere complete medium and seeded to a 96-well flat-bottom suspension plate. The 100 µL of Neurosphere complete medium was adjusted to different glucose concentrations: 100% (450 mg/dL), 50% (225 mg/dL), 25% (112.5 mg/dL), 10% (45 mg/dL), and 0% (0 mg/dL) of standard cell culture glucose concentration using Gibco glucose solution 200 g/L (Gibco BRL) and cultured for 2, 24, and 48 h. Cell viability was determined using the thiazolyl blue tetrazolium bromide assay (MTT, Sigma-Aldrich). The samples were measured on a Paradigm™ multiplate reader (Beckman Coulter, Brea, CA, USA) at wavelengths of 570 nm and 650 nm.

2.3. Invasion Assay

The ability of glucose-starved GSCs to invade through a Matrigel-coated membrane was evaluated using a modified Boyden chamber assay, as previously described in reference [18]. The inserts (Falcon) were coated with Matrigel before cells (1×10^5/500 µL) were resuspended in Neurobasal complete medium either with or without glucose. The inserts were placed in a 24-well plate and coated with 500 µL of growth factor-reduced Matrigel (BD, Franklin Lakes, NJ, USA) diluted in Neurobasal glucose-depleted medium (1:100). The bottom of the 24-well plate was also coated. Following an incubation period of 1 h at 37 °C, the Matrigel was removed, the cells were seeded into the inserts, and the

chambers were filled with 800 µL Neurobasal complete medium containing glucose at a standard concentration (450 mg/dL) and 10% FBS. After 48 h, the cell suspensions inside the inserts were removed and the non-invaded cells on the inner membrane of the inserts were cautiously stripped off with a cotton swap drenched in PBS. Next, cells that invaded through the membrane were fixed by adding ice-cold methanol for 10 min. The inserts were washed twice with PBS and cells were stained with hematoxylin for 5 min. Images were taken on a Zeiss microscope (objective: Zeiss Plan S 1.0 × FWD 81 mm), and the number of cells invaded was evaluated with ImageJ 1.8.0. (Rasband, W.S., U.S. National Institutes of Health, Bethesda, MD, USA).

2.4. Soft Agar Colony Formation Assay

The clonogenic capacity of GSCs was determined as previously described in reference [17]. Briefly, six-well plates were coated with 1.5 mL of 1% agarose (Life Technologies) in Neurobasal medium (as a bottom layer) and incubated at 37 °C for 1 h. Then, the middle layer containing 0.6% agarose, 5×10^3 cells/well in Neurobasal complete medium (with and without 10% standard glucose concentration) was added. After solidification at room temperature (RT) for 1 h, the top layer was then prepared and incubated at 37 °C and 5% CO_2 for 4 weeks. Subsequently, 1 mg/mL 4-nitro-tetrazolium chloride (NBT) solution (Sigma-Aldrich) in PBS was added overnight (37 °C) to stain the colonies. ImageJ 1.8.0. (Rasband, W.S., U.S. National Institutes of Health, Bethesda, MD, USA) was used to count the colonies.

2.5. Luciferase Reporter Assay

The luciferase reporter assay was performed as previously described in reference [19]. After transfection of GSCs with a stable lentiviral reporter construct comprising seven *TCF* binding sites trailed by a luciferase cassette, puromycin selection (2 µg/mL) was performed. Subsequently, transfected cells were cultured under standard glucose concentrations and glucose deprivation (48 h). The emitted luminescence was measured at a wavelength of 490 nm on a Paradigm™ multiplate reader (Beckman Coulter, Brea, CA, USA) and normalized to β-galactosidase activity.

2.6. Western Blot

Cells were washed with PBS and lysed in ice-cold RIPA buffer. Protein concentrations were quantified using the DC Protein Assay Kit (BioRad, Hercules, CA, USA) and readout was performed using the Paradigm™ Multiplate Reader (Beckman Coulter, Brea, CA, USA) at a wavelength of 750 nm. The subsequent steps were performed as previously described in reference [20]. Primary antibodies: active β-catenin 1:1000 (non-phospho/active β-catenin, Ser33/37/Thr41, rabbit mAb, Cell Signaling, Danvers, MA, USA) and GAPDH 1:5000 (GAPDH, D4C6R, mouse mAb; Cell Signaling, Danvers, MA) were diluted in 5% BSA and incubated overnight at 4 °C on the membranes. Secondary antibodies: goat anti-rabbit IRDye800CW (1:10,000, LI-COR #926-32211) and goat anti-mouse IRDye680RD (1:10,000, LI-COR #926-68070) were diluted in 5% BSA and incubated for 1 h at RT. The fluorescence was assessed using LI-COR Odyssey CLx imager followed by densitometry. GAPDH was used as a housekeeping protein for normalization. Original western blot images can be found in the Supplementary Materials.

2.7. Immunostaining for Active β-Catenin

Immunostaining for active β-catenin was performed in standard cell culture conditions (450 mg/dL) and under glucose deprivation for 48 h. Briefly, we harvested the cells (10,000 cells/200 µL), counted, washed thoroughly with PBS, and centrifuged on microscope slides using a Cytospin. Samples were then dried, fixated in 4% PFA, incubated in Tween 20 and blocked using 5% BSA. Primary antibody was incubated over night at 4 °C (active β-catenin 1:1000 in 5% BSA/TBST (non-phospho/active β-catenin, Ser33/37/Thr41, rabbit mAb; Cell Signaling, Danvers, MA, USA)). Subsequently, the samples were washed thoroughly using

TBST and incubated for 1 h at room temperature with a secondary antibody goat anti-rabbit IRDye800CW (1:10,000, LI-COR #926-32211)). The cells were stained using DAPI, pictures were taken with a fluorescence microscope (Axiovision Apotome. Two-confocal microscope (Zeiss, Jena, Germnay)) and assessed by the software ZEN. The assessed fluorescence signals (green) translated into the amount of stained active β-catenin.

2.8. Quantitative Real-Time PCR (RT qPCR)

RNA isolation was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA synthesis using the M-MLV reverse transcriptase (Promega, Madison, WI, USA), M-MLV buffer (Promega), random hexamer primers, and Ribolock for RT-qPCR. SYBR Green Supermix (BioRad, Hercules, CA, USA), 10 ng cDNA, and 10 pmol primers were combined and run in a CFX Connect thermal cycler (BioRad). The expression of target genes was normalized to beta-2-microglobulin. Primer sequences used in this study were as follows: *β-catenin*: Fwd-GGGCCTCAGAGAGCTGAGTA, Rev-TGAGCAGCATCAAACTGTGTAG; *Axin2*: Fwd- AGCCAAAGCGATCTACAAAAGG, Rev-GGTAGGCATTTTCCTCCATCAC; *ZEB1*: Fwd-AAGAATTCACAGTGGAGAGAAGCCA, Rev-CGTTTCTTGCAGTTTGGGATT; *C-Myc*: Fwd-CCTTAATTAAAATGCCCCTCAACGTTAGCT, Rev- GGAATTCCATATGTTACGCACAAGAGTTCCGTA; and *MCT-1*: Fwd- GCTGGGCAGTGGTAATTGGA, Rev-CAGTAATTGATTTGGGAAATGCAT.

2.9. Whole Transcriptome Analysis

To obtain differential expression of genes, RNA was extracted and whole transcriptome analysis (3′ mRNA sequencing) was performed at the NGS Core Facility (Bonn, Germany). We applied the R package Deseq2 to identify differentially expressed genes in the control group versus the test/inhibitor group (dimethyl sulfoxide (DMSO) and/or LGK974) and used the R package clusterProfiler to recognize differentially expressed genes enriched in the KEGG pathways. Since DMSO alone has been shown to influence gene expression [21,22], we first attempted to exclude those genes which were primarily affected by the DMSO treatment. For this, we first filtered out the altered genes from the datasets that were only associated with DMSO treatment (standard glucose concentrations (Glc+) versus glucose withdrawal (Glc−). Subsequently, the obtained values were compared to the LGK974 treatment datasets (Glc+ versus Glc−).

2.10. LGK974 and DMSO Treatment

We assessed the viability of GSCs cultivated in glucose-depleted conditions and in the presence of the pharmacological WNT inhibitor LGK974 (a porcupine inhibitor (no. 1241454); Peprotech, Hamburg, Germany). LGK974 was dissolved in DMSO (Sigma-Aldrich) and used according to the manufacturer's instructions. Specifically, we performed MTT assays on GSCs grown with and without glucose (450 and 0 mg/dL, respectively) and treated with five different LGK974 concentrations (GBM1 and JHH520: 20 μM, 10 μM, 5 μM, 2.5 μM, and 1.25 μM; BTSC233: 80 μM, 40 μM, 20 μM, 10 μM, and 5 μM). DMSO solvent controls (Glc+/Glc−) were used for normalization. We normalized the treatment groups to their corresponding controls after a 48 h incubation period.

2.11. In Vitro Drug Screening

An in vitro drug screen was conducted as previously reported in reference [23]. Using the Tecan D300e Digital Dispenser, a drug library, comprising 231 clinically approved chemotherapeutic drugs, was distributed in 384-well plates (Corning, Corning, NY, USA; Tecan, Männedorf, Switzerland). To determine the optimal number of cells to use in the screen, the growth curves of each cell line were analyzed before seeding them into the drug-coated plates. Cell viability was assessed by a GUAVA MUSE cytometer (Count and Viability, Luminex, Austin, TX, USA). Subsequently, an aliquot of 30 μL of cell suspension was dispensed into the prepared 384-well plates and incubated for 72 h. The CellTiter-Glo

luminescent cell viability assay (Promega, Germany) was used to determine cell viability according to the manufacturer's protocol and readout.

2.12. Gas Chromatography Mass Spectrometry: Cell Harvesting and Metabolite Extraction

Cells were cultivated under standard glucose concentration (450 mg/dL) (37 °C, 5% CO_2) and washed twice with PBS before glucose-free Neurobasal medium was added to induce starvation. Starved cells were treated with LGK974 or DMSO for 48 h before being harvested by centrifugation (4 °C, 5 min, and 1000 rcf), counted, and washed with ice-cold 0.9% (*w*/*v*) sodium chloride (NaCl) solution. For cell disruption and metabolite extraction, 350 µL of a methanol: chloroform (10:4.28) solution was added. The samples were vortexed and incubated for 1 h at −20 °C. Subsequently, 560 µL of water containing the internal standard (5 µM ribitol) was added and the samples were vortexed and incubated on ice for 10 min. Samples were then centrifugated at maximum speed (12,000 rpm) for 2 min and two phases were obtained (an aqueous upper phase and a hydrophobic lower phase). The upper aqueous phase was dried by lyophilization. After resuspension in 250 µL methanol (50%), an aliquot of 50 µL was dried in a glass inlet for analysis by gas chromatography.

2.13. Gas Chromatography Mass Spectrometry

The samples were prepared and analyzed by gas chromatography mass spectrometry (GC-MS) analysis as previously reported in references [24,25]. Metabolites were identified by comparing obtained spectra to spectra in the NIST14 Mass Spectral Library (https://www.nist.gov/srd/nist-standard-reference-database-1a-v14) (retrieved on 10 May 2021) using the MassHunter Qualitative program (v.b08.00; Agilent Technologies, Santa Clara, CA, USA). In addition, a quality control sample including all target substances was analyzed. The MassHunter Quantitative program was used to combine the peaks (v.b08.00; Agilent Technologies). For relative quantitation, all metabolite peak areas were normalized to the peak area of the internal standard ribitol. A defined dose of LGK974 was used in all cell lines (GBM1: 10 µM, JHH520: 20 µM, BTSC233: 40 µM) in these experiments.

2.14. Statistical Analyses

All statistical tests were performed using unpaired Student's *t*-tests using GraphPad-Prism software, version 8.0 (GraphPad Software, San Diego, CA, USA). All results are presented as mean + SD of a minimum of three independent biological replicates. For all experiments, significance was defined as a p value below 0.05.

3. Results

3.1. Glucose Starvation Impacts Cell Viability and Invasive Potential of GSCs

First, we cultured GSCs in a Neurobasal medium containing glucose at varying concentrations (450 mg/dL, 225 mg/dL, 112.5 mg/dL, and 45 mg/dL) for 24 and 48 h (Figure 1A). In GBM1 and JHH520 cells, reduction in glucose levels (450 mg/dL–45 mg/dL) did not significantly affect cell viability. However, reducing the glucose concentration to 45 mg/dL significantly decreased the viability of BTSC233 cells ($p < 0.05$). When compared to the control (non-starved cells cultured at a standard glucose concentration of 450 mg/dL), reduced glucose levels showed little effect; however, complete glucose deprivation significantly reduced GSC's viability. In addition, glucose-depleted cultures of JHH520 and BTSC233 cells displayed significantly enhanced invasion after 48 h compared to the control (JHH520 $p < 0.05$, BTSC233 $p < 0.001$) (Figure 1B). Interestingly, all cell lines showed a significant increase in clonogenic capacity when cultivated in reduced glucose concentration media (45 mg/dL) as opposed to standard cell culture conditions (450 mg/dL glucose) (GBM1 $p < 0.001$, JHH520 $p < 0.01$, and BTSC233 $p < 0.05$) (Supplementary Figure S1A).

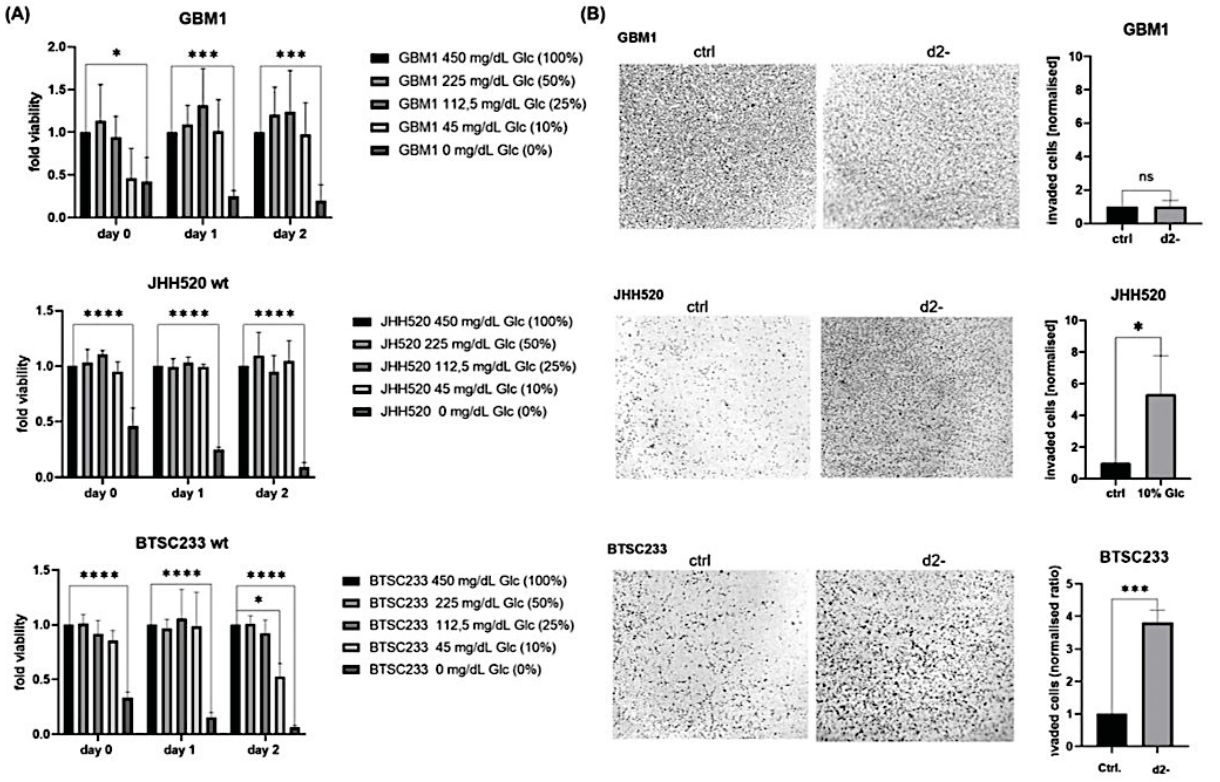

Figure 1. Glucose starvation decreases cell viability and enhances invasion in glioblastoma cell lines. (**A**) Glioblastoma cell lines (GBM1, JHH520, and BTSC233) were cultivated in standard cell culture glucose concentrations (450 mg/dL), decreased glucose concentrations (225 mg/dL, 112, 5 mg/dL, and 45 mg/dL) and in a glucose-depleted cell culture medium. The glucose concentration reduced to 45 mg/dL significantly decreased cell viability ($p < 0.05$) in BTSC233, whereas glucose depletion significantly inhibited viability in all cell lines ($p < 0.0001$). (**B**) A Boyden chamber assay was performed with all cell lines (GBM1, JHH520, and BTSC233) cultivated in standard (450 mg/dL) as a control condition (ctrl) and were compared to the corresponding starved samples (0 mg/dL) for 48 h (d2-). Glucose deprivation significantly enhanced invasion in JHH520 ($p < 0.05$) and BTSC233 cells ($p < 0.001$). The data are represented as mean + SD ($n = 3$). Statistical significance was calculated using an unpaired Student's *t*-test. * $p < 0.05$, *** $p < 0.001$, and **** $p < 0.0001$.

3.2. Glucose Starvation Enhances WNT Activity and Induces Alterations in β-Catenin and Associated Genes

WNT signaling plays an important role in mediating metabolic resistance in neoplastic tissues [14]. Therefore, we assessed the activity of the canonical WNT pathway in GSCs using the *TCF* luciferase reporter assay. A significant increase in luciferase signal intensity was observed in *TCF* luciferase-transfected GBM1 and JHH520 cells after 48 h of glucose withdrawal compared to controls (non-starved *TCF* luciferase-transfected cells) ($p < 0.05$). No change was observed in BTSC233 cells (Supplementary Figure S1B). The findings in GBM1 and JHH520 cells suggested that glucose deprivation in GSCs may be associated with the activation of the canonical WNT pathway. Therefore, we investigated the direct effect of glucose deprivation (24, 48 h) on β-catenin protein levels in wildtype GSCs. We observed significantly increased levels of β-catenin in JHH520 cells after 24 h of starvation ($p < 0.05$) (Supplementary Figure S1C). Both starved GBM1 and BTSC233 cells showed no significant change in β-catenin protein levels compared to the control group. Since

transcription-related changes can be more sensitive compared to stable/conserved protein levels, we extended our analysis by evaluating the mRNA expression of β-catenin and WNT/β-catenin downstream genes (*AXIN2*, *ZEB1*, *MYC*, and *MCT1*) after 24 and 48 h of glucose deprivation (Figure 2). In GBM1 cells, we detected significantly upregulated *CTNNB1* (β-catenin), *AXIN2*, *ZEB1*, and *MYC* mRNA expression levels after starvation for 48 h ($p < 0.01$). A 24 h period of starvation in GBM1 cells also induced a significant upregulation of *CTNNB1*, *AXIN2*, and *MYC* mRNA expression, but not of *ZEB1* transcripts. It was also found that *ZEB1* was significantly downregulated (24 h of treatment), while *MYC* was upregulated at the mRNA level in JHH520 cells (48 h). BTSC233 cells did not show alterations in the mRNA expression of these selected genes. The transcript levels of *MCT1* were not altered in any of these cell lines.

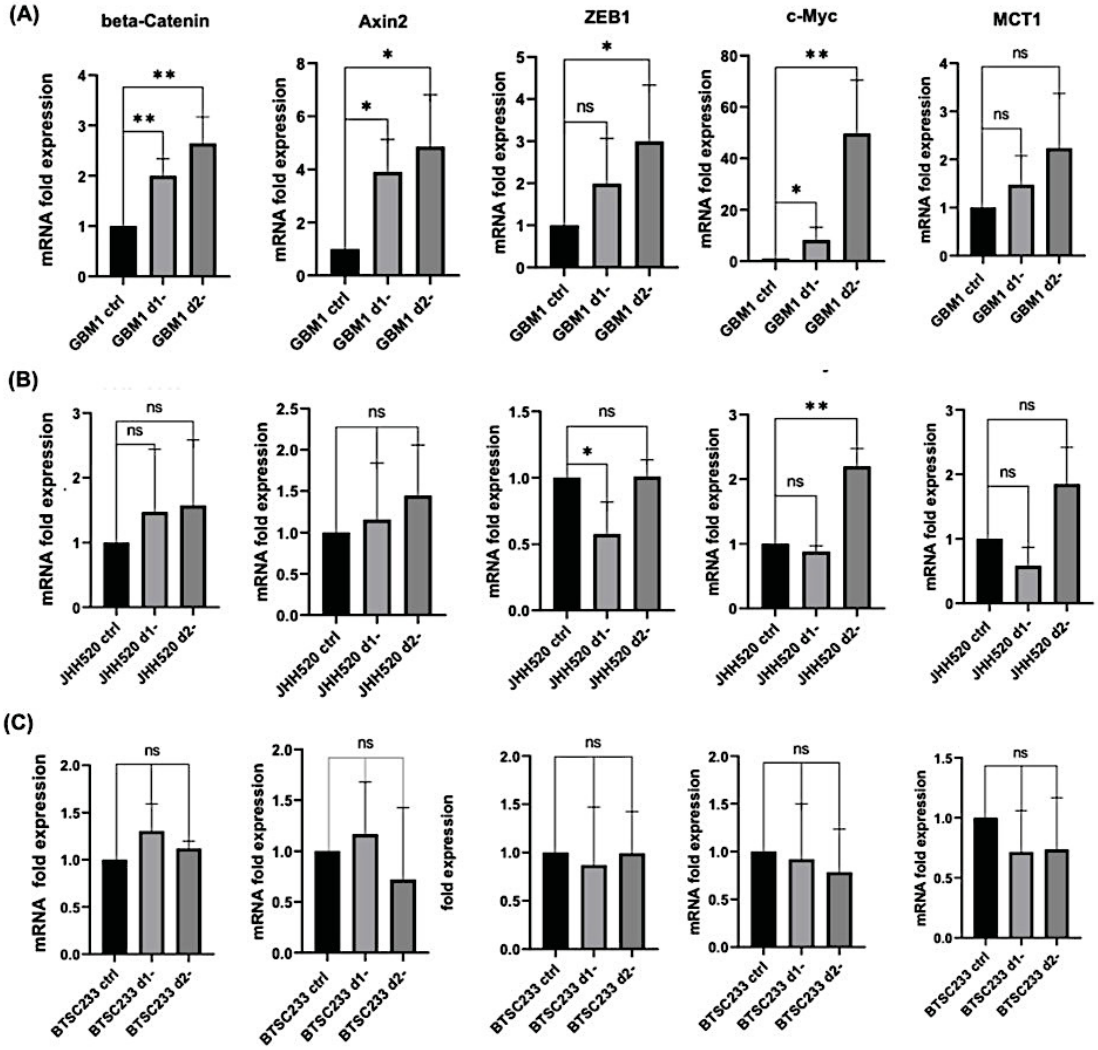

Figure 2. Differential mRNA expression of *CTNNB1* and β-catenin target genes in glucose-starved GSCs. (**A–C**) Differential mRNA expression of β-catenin and downstream genes of WNT signaling were assessed in three GSCs: GBM1, JHH520, and BTSC233 after 24 h (d1-) and 48 h (d2-) of glucose starvation. The data are presented as mean + SD ($n = 3$). Statistical significance was calculated using an unpaired Student's *t*-test. * $p < 0.05$, ** $p < 0.01$.

3.3. Pharmacological WNT Inhibition Sensitizes GSCs to Glucose Starvation-Induced Cell Death and Moderately Affects Gene Expression of β-Catenin and Associated Genes

Next, we assessed the viability of cell lines cultivated in glucose-depleted conditions and under pharmacological WNT inhibition with LGK974 (Figure 3A). In JHH520 cells, treatment with LGK974 (10 μM and 20 μM) significantly decreased the viability of glucose-depleted cells ($p < 0.01$). In BTSC233 cells, high concentrations of LGK974 (80 μM, 40 μM, and 20 μM) and glucose starvation significantly reduced cell viability ($p < 0.01$–$p < 0.0001$), suggesting that WNT signaling mediates metabolic resilience to maintain viability under nutrient/metabolic stress. Transcriptional changes in WNT/β-catenin target genes (*AXIN2*, *ZEB1*) were observed in JHH520 and BTSC233 cells ($p < 0.05$) upon treatment with LGK974 or control (DMSO) and simultaneous glucose deprivation (Figure 3B).

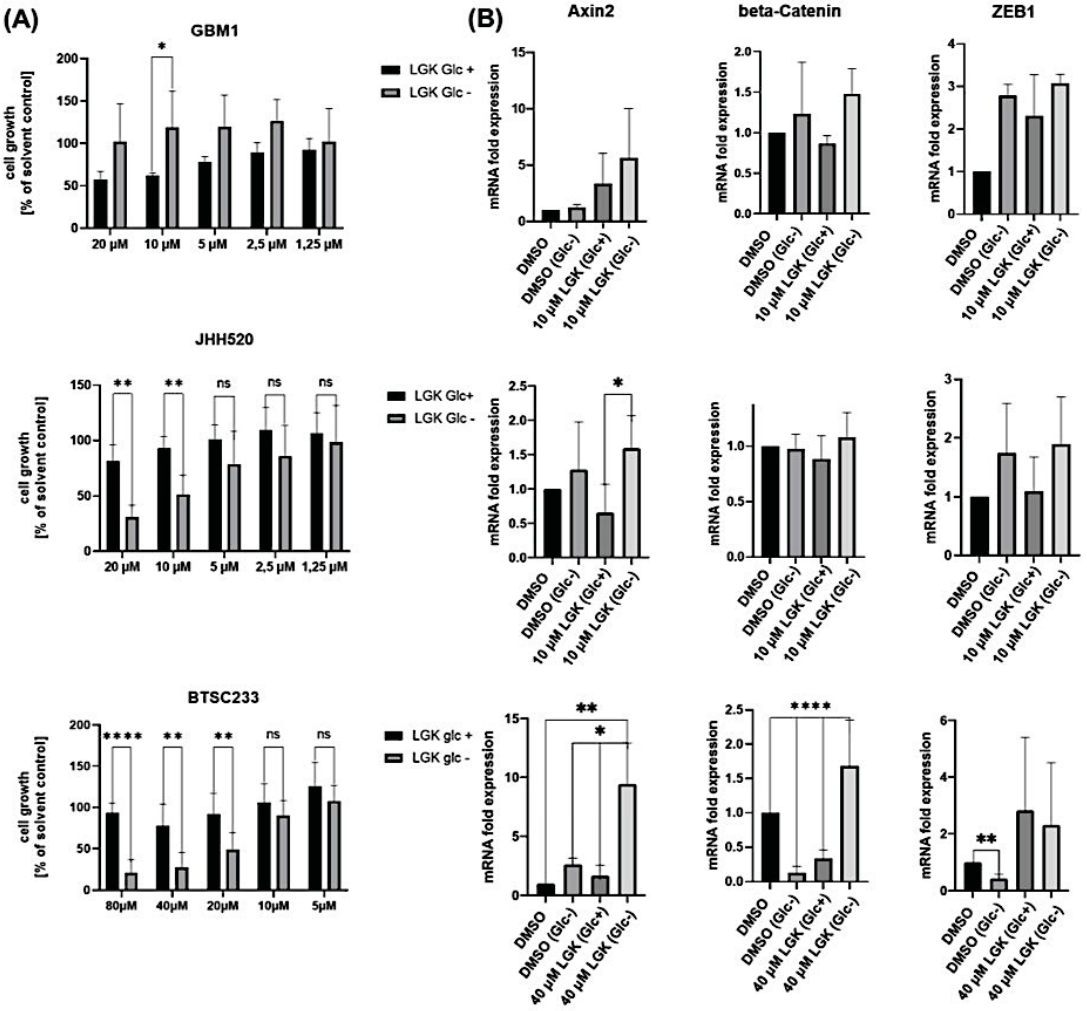

Figure 3. LGK974 sensitizes GSCs to glucose starvation-induced cell death and affects the mRNA expression of *CTNNB1* and associated genes *AXIN-2* and *ZEB1*. (**A**) We assessed the viability of our GSC lines cultured in standard glucose concentrations of 450 mg/dL (Glc+) or in a glucose-depleted standard cell culture medium of 0 mg/dL (Glc−) and simultaneously performed the pharmacological inhibition of WNT signaling with LGK974 (LGK) for a time period of 48 h. DMSO solvent controls were used for normalization. In order to normalize to the solvent control, equivalent amounts of DMSO were used in all conditions per cell line. LGK974 significantly decreased the viability of

glucose-deprived JHH520 cells at 10 μM and 20 μM ($p < 0.01$), and at 20 μM, 40 μM ($p < 0.01$), and 80 μM ($p < 0.0001$) in BTSC233 compared to cells that have been treated with LGK974 but which have not been depleted of glucose (LGK Glc+). GBM1 glucose-starved cells that were simultaneously treated with LGK974 displayed increased viability ($p < 0.01$) when treated with 10 μM LGK974 compared to cells that have been treated with LGK974 but which have not been depleted of glucose (LGK Glc+). (**B**) We also assessed the mRNA expression of *AXIN2*, *CTNNB1* (β-Catenin), and *ZEB1* of our GSC lines GBM1, JHH520, and BTSC233 after a time period of 48 h of treatment with DMSO (equivalent in all conditions per cell line) and in a standard glucose concentration of 450 mg/dL as a control (DMSO). Additionally, cells have been depleted of glucose and cultivated in DMSO (DMSO Glc−), treated with LGK974 (LGK) in defined (assessed concentrations inducing significant change of viability when depleted of glucose per cell line: GBM1: 10 μM LGK974; JHH520: 10 μM LGK974; and BTSC233: 40 μM LGK974) standard glucose concentrations (LGK Glc+) and under glucose withdrawal (LGK Glc−). *AXIN2* mRNA levels were significantly increased in JHH520 and BTSC233 cells treated with LGK974 and depleted of glucose ($p < 0.05$–$p < 0.01$). The data are presented as mean + SD ($n = 3$). Statistical significance was calculated using an unpaired Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$.

To obtain a comprehensive overview of the transcriptional changes beyond WNT/β-catenin target genes, we next performed a genome-wide transcriptional analysis. When comparing samples treated with DMSO (Glc+/−) and/or LGK974 (Glc+/−), both upregulated and downregulated gene clusters were found in all three cell lines (Figure 4A,B). Among them, significantly altered genes were: GBM1: up-regulated genes: *RYR1*, *HS6ST2*, and *C14orf132*, down-regulated genes: *TACSTD2*, *VAMP8*, and *BPIFA1*; JHH520: up-regulated genes: *PAWR*, *TIMP3*, and *HOXB7*, down-regulated genes: *ABCG2*, *TSACC*, and *PYY*; BTSC233: upregulated genes: *NCAN*, *SHC2*, and *NF1*, downregulated genes: *SDK1*, *TFPI2*, and *NBDY*. Furthermore, KEGG pathway analysis showed that these differentially expressed genes (DEGs) were highly associated with pathways ranging from metabolism to cancer (Figure 4C). Of interest, we also identified DEGs within the WNT/β-catenin pathway that were induced by glucose deprivation (Supplementary Figure S2). In addition, we evaluated the expression of β-catenin-dependent and independent target genes, retrieved from a recent study [26], and found them altered in our datasets (Supplementary Figure S3).

We next performed immunostaining for active β-catenin in GSCs (GBM1, BTSC233) under glucose deprivation for 48 h (Supplementary Figure S4). Staining increased in cells deprived of glucose, indicating a quantitatively higher amount of transcriptionally active β-catenin. Likewise, *TCF* luciferase activity was increased significantly in GBM1 and JHH520 cells after 48 h of glucose deprivation, whereas a tendency toward the upregulation of *TCF* luciferase activity was observed in BTSC233 cells.

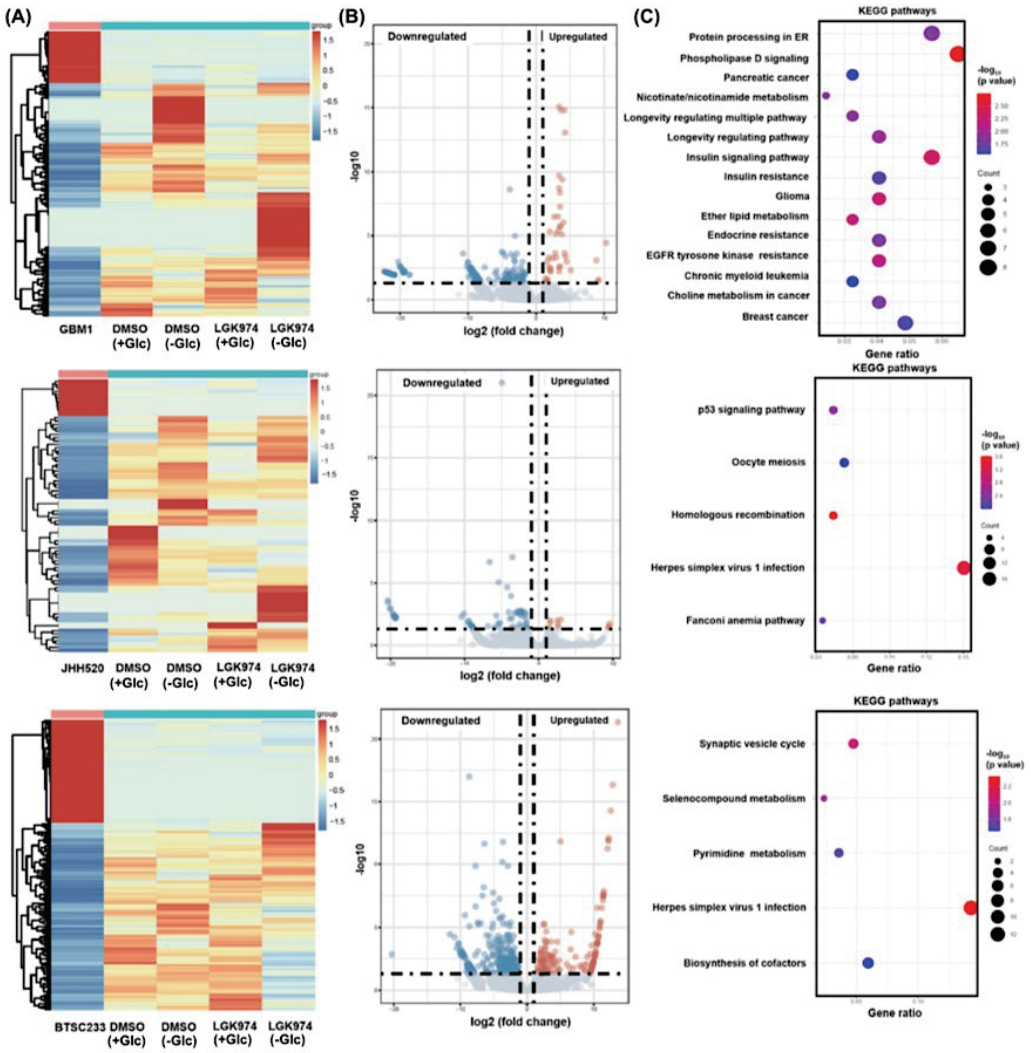

Figure 4. Transcriptome-wide gene expression analysis of GBM1, JHH520, and BTSC233 cells. (**A**) A heatmap showing variations in gene expression in cells treated with DMSO as solvent control and LGK974 in standard cell culture glucose concentration (450 mg/dL) and under complete glucose deprivation. (**B**) Altered genes in treated compared to untreated cell lines and (**C**) KEGG-associated pathways.

3.4. Treatment with LGK974 and/or Glucose Starvation Alters Intracellular Metabolite Concentrations

Next, we performed gas chromatography mass spectrometry (GC-MS) to analyze intracellular metabolites in GSC lines treated with LGK974 or DMSO for a time period of 48 h under standard (450 mg/dL) and depleted glucose concentrations (0 mg/dL) (Figure 5). The intracellular level of the glucogenic amino acid alanine was significantly ($p < 0.0001$–$p < 0.05$) reduced under glucose deprivation in our GSC lines irrespective of LGK974 treatment (Figure 5A). We also observed a reduction of valine (GBM1 $p < 0.05$, JHH520 $p = 0.67$, and BTSC233 $p < 0.05$). Other glucogenic amino acids such as glutamate, 5-oxoproline, methionine, and aspartate showed no changes. Treatment with LGK974 combined with glucose deprivation impacted ketogenic amino acids (leucine, lysine, phenylalanine, isoleucine, and threonine) (Figure 5B). Leucine (in BTSC233 $p < 0.05$) and lysine

(JHH520: $p = 0.051$, BTSC233: $p = 0.058$) were found to be reduced upon LGK974 treatment alone. Isoleucine was reduced upon LGK974 treatment in BTSC233 cells ($p < 0.05$), while simultaneous WNT inhibition and glucose deprivation resulted in the reduction of isoleucine in all cell lines (GBM1 and BTSC233: $p < 0.05$; JHH520: $p = 0.099$). Both phenylalanine and threonine were not changed significantly.

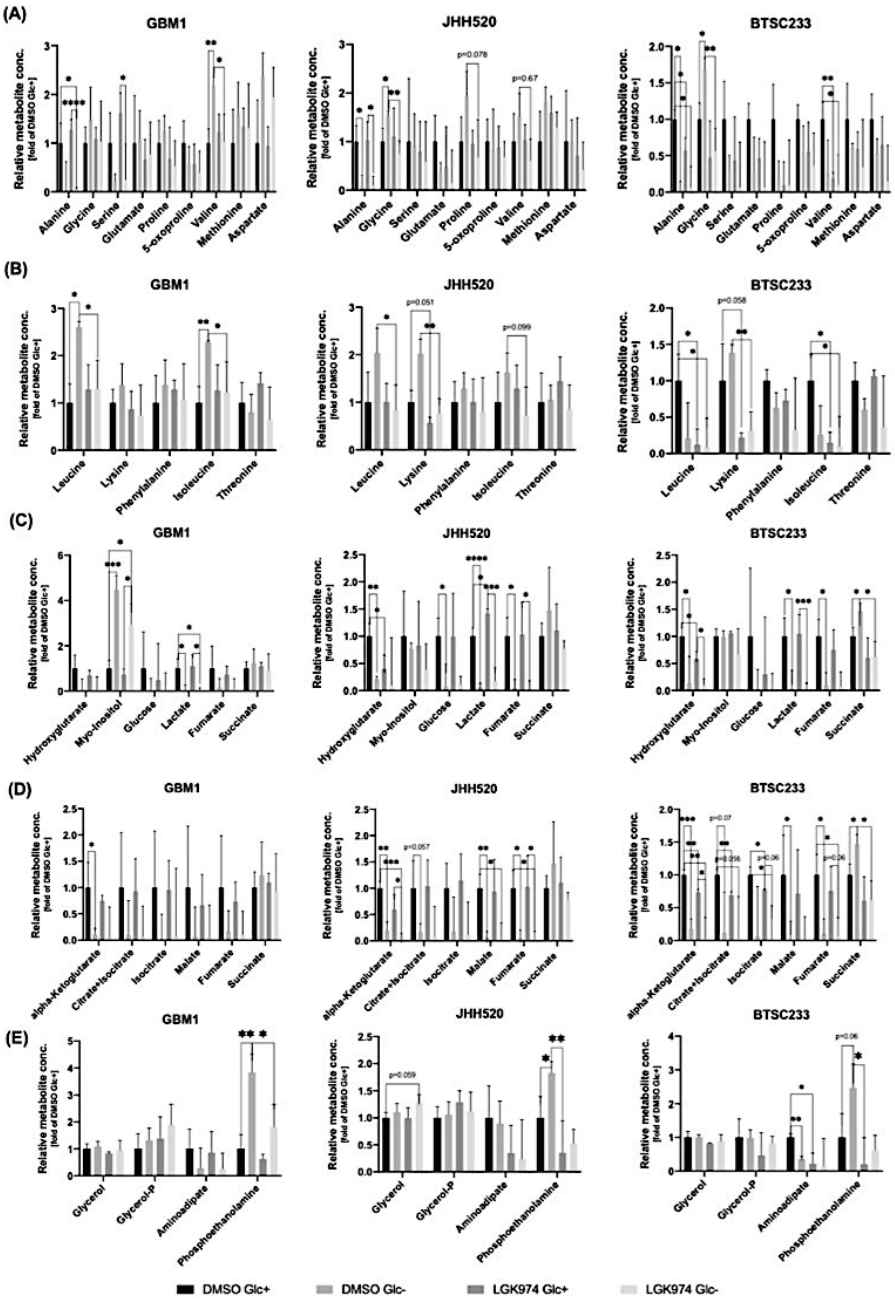

Figure 5. LGK974 treatment and/or glucose starvation-altered intracellular metabolite concentrations as determined by GC-MS. The effect on intracellular metabolite concentrations in cells treated with DMSO and LGK974 in standard cell culture glucose concentration (450 mg/dL) (DMSO Glc+ (as a solvent control) and LGK974 Glc+) and under complete glucose deprivation for a time period of 48 h

(DMSO Glc− and LGK974 Glc−) is shown. Quantitative characterization of diverse intracellular metabolites such as (**A**) glucogenic amino acids (alanine, glycine, serine, glutamate, (5-oxo-)proline, valine, methionine, and aspartate), (**B**) ketogenic amino acids (leucine, lysine, phenylalanine, isoleucine, and threonine), (**C**) oncometabolites (hydroxyglutarate, myo-inositol, glucose, lactate, fumarate, and succinate), (**D**) tricarboxylic acid (TCA) metabolites (alpha-ketoglutarate, citrate, isocitrate, malate, fumarate, and succinate) and (**E**) lipophilic metabolites (glycerol(-P), aminoadipate, and phosphoethanolamine) were evaluated. The data are presented as mean + SD (n = 3). The y-axis depicts the relative metabolite concentration in the intracellular compartment (normalized to the corresponding solvent control (DMSO Glc+)). Statistical significance was calculated using an unpaired Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

WNT inhibition by LGK974 also reduced the intracellular concentration of the oncometabolite hydroxyglutarate, particularly in JHH520 cells ($p < 0.0001$) and BTSC233 cells ($p < 0.05$) (Figure 5C). Succinate levels were increased by DMSO and glucose deprivation in BTSC233 cells; however, the combined use of LGK974 and glucose starvation resulted in the suppression of succinate levels. Starvation resulted in decreased intracellular levels of glucose, lactate, and fumarate in JHH520 cells ($p < 0.0001$) and BTSC233 cells ($p < 0.05$), irrespectively of LGK974 treatment. Myoinositol, a well-known oncometabolite in glioblastoma, was significantly elevated in starved GBM1 cells ($p < 0.001$). Glucose starvation also led to significantly reduced levels of tricarboxylic acid (TCA) metabolites, such as alpha-ketoglutarate, in GSCs treated with DMSO (GBM1: $p < 0.01$, JHH520: $p < 0.01$, and BTSC233: $p < 0.05$) and LGK974 (JHH520: $p < 0.05$, BTSC233: $p < 0.05$) (Figure 5D). Some TCA metabolites (citrate, isocitrate, malate, and fumarate) were significantly reduced after the glucose starvation of JHH520 and BTSC233 cells ($p < 0.001$–$p = 0.057$). Glucose deprivation led to an increased intracellular level of phosphoethanolamine in all cell lines ($p < 0.0001$–$p < 0.001$), irrespectively of WNT inhibition (Figure 5E).

3.5. In Vitro Drug Screen in Glucose-Deprived GSCs

To determine the effects of nutritional stress on chemosensitivity, we performed an in vitro drug screen comprising 231 proven chemotherapeutic drugs using two GSCs enriched cell lines (GBM1, BTSC233) grown in standard cell culture conditions and under glucose deprivation (Supplementary Figure S5). Staurosporin, a global kinases inhibitor was used as a positive control. The analysis showed that eleven drugs sensitized the GSCs to starvation-induced cell death—including LGK974 and berberine, which both impair the WNT pathway. In addition to these, disulfiram (aldehyde dehydrogenase inhibitor), andrographolide (NFkB inhibitor), auranofin bacterial (inhibitor of bacterial thioredoxin reductase), pazopanib (multitargeted tyrosine kinase inhibitor), entinostat (histone deacetylase inhibitor), honokiol (ERK inhibitor), ravoxertinib (ERK inhibitor), rigosertib sodium (PI3K- and Polo-like Kinase inhibitor), and masitinib mesylate (c-Kit, FGFR, PDGFR, Scr inhibitor) were also identified as drugs that sensitize to starvation-induced cell death.

4. Discussion

Although aberrant WNT activation has been associated with malignant transformation in various cancers, its role in glioblastoma is yet to be fully unveiled. WNT signaling mediates the clonogenicity and growth of neural progenitor cells and mediates chemoresistance to alkylating agents such as Temozolomide (TMZ) in glioblastoma [27]. Due to its role in promoting metabolic plasticity, WNT upregulation has been postulated as a mediator of metabolic resilience in malignant tissues [28]. Thus, our study focused on the WNT-mediated response to glucose deprivation in GSCs. Initially, we assessed the viability of glucose-starved GSCs by performing an MTT assay, thereby determining the percentage of cells surviving extreme metabolic stress. A small subpopulation of cells managed to survive in a glucose-deprived microenvironment (Figure 1). In our next experiment, we assessed invasive capacities after glucose starvation and showed that the depletion of

glucose led to the acquisition of an invasive phenotype, possibly by inducing a mesenchymal transition (Figure 3). This acquisition of an invasive phenotype in nutrient-limiting microenvironments has been shown across various cancer tissues before [29].

Furthermore, we performed a soft agar colony formation assay and quantified the self-renewal properties as a surrogate of the stem-like cell phenotype, observing the increased clonogenic potential of GSCs that were exposed to low glucose concentrations for four weeks. This indicates an enrichment of GSCs by nutrient deprivation. A similar advantage in survival leading to the selection of stem-like cancer cells has already been observed after chemo- and radiotherapy [30]. The impact of glucose starvation in our analysis was also evident on both the local (*β-catenin* and target genes) and the global (whole-genome transcriptome) genomic level. Moreover, the significant increase in luciferase signal also suggested an increased activity of the canonical WNT pathway following glucose starvation. Based on our observations, we concluded that glucose-depleted GSCs activate canonical WNT signaling in response to metabolic stress. Indeed, simultaneous WNT inhibition with the porcupine inhibitor LGK974, which interferes with the endoplasmatic release of WNT molecules, led to significantly decreased viability under glucose starvation in a dose-dependent manner. Although this phenomenon can predominantly be attributed to direct WNT/β-catenin inhibition, off-target effects of LGK974 cannot be excluded. Furthermore, heterogenic transcriptional characteristics of GSCs used in this study (GBM1: adult male, classical, TP53 p.L130I, IDH-wildtype; JHH520: adult female, mesenchymal, TP53 p.H179D, IDH-wildtype; and BTSC233: adult female, mesenchymal, IDH-wildtype) may contribute to minor differences in observed responses towards metabolic stress. Such inherited heterogeneity between cancer cell lines leading to experimental discrepancies has previously been discussed [31].

In vitro drug screening identified LGK974 and berberine (WNT inhibitor used for hypercholesterinemia, diabetes, and hypertension [32]) as drugs that sensitize cells to starvation-induced cell death. In addition to that, we characterized the metabolic profile under glucose starvation, WNT inhibition, and a combination of WNT inhibition and glucose starvation, by utilizing GC-MS. We observed significant changes in various metabolites under WNT inhibition alone and in combination with glucose depletion. The glucogenic amino acid alanine was significantly reduced in starved cell lines, most likely due to anaplerosis [33], whereas other glucogenic amino acids (such as glutamate, 5-oxoproline, methionine, and aspartate) showed no changes. Whether these effects are confined to the metabolic microenvironment of gliomas or common to other cancers requires further studies. Notably, in all cell lines, ketogenic amino acids lysine and leucine were reduced, even more significantly under simultaneous WNT inhibition and glucose starvation. This indicates an additional role of WNT signaling in ketogenesis by fatty acid oxidation, which has also previously been reported [34]. Our results contribute to a more comprehensive understanding of WNT signaling as an important player in the regulation of ketogenesis not only through the beta-oxidation of fatty acids but also by the utilization of ketogenic amino acids. The impaired survival of glucose-deprived GSCs by simultaneous WNT inhibition may be due to the limited availability of ketogenic amino acids for the consequent utilization of anaplerotic reactions in the tricarboxylic acid cycle. In particular, alpha-ketoglutarate, citrate and isocitrate, malate, and fumarate were decreased in starved GSCs independent of WNT inhibition, suggesting a general mechanism, such as the rapid incorporation into the reaction chains of the tricarboxylic acid cycle. In addition, WNT inhibition of glucose-deprived GSCs affected several oncometabolites, such as hydroxyglutarate, myo-inositol, succinate, and fumarate. Hydroxyglutarate is an oncometabolite that accumulates in IDH-mutant glioma cells and correlates with poor prognosis [35]. In our study, hydroxyglutarate was significantly reduced under LGK974 treatment, suggesting a role of the WNT pathway in regulating oncometabolic-driven cancer growth. WNT inhibition decreased the phosphoethanolamine concentration, which was elevated by glucose starvation. A recent study showed that mutant IDH1 gliomas downregulate the synthesis of phosphocholine and phosphoethanolamine in a 2-hydroxyglutarate-dependent man-

ner [36]. Although WNT activation played an important role in maintaining cell survival under extreme nutrient restriction, the exact mechanism by which glucose deprivation can enhance β-catenin activity remains unclear. Previously, it has been discussed that the degradation of β-catenin upon glucose deprivation is GSK3β-independent and mainly involves the protein kinase C (PKC)-dependent pathway [37]. Likewise, AMP-activated protein kinase (AMPK) phosphorylates β-catenin at Ser 552 [38] and further regulates its transcriptional level via phosphorylated histone deacetylase 5 (HDAC5) [39].

In summary, our findings reveal novel pleiotropic effects of WNT signaling on metabolic activity in glioblastoma stem-like cell lines. Metabolic fingerprints might possibly complement classic biomarkers, allowing for the better prediction of tumor behavior and clinical prognosis. Considering that in vitro tumor models do not closely mimic the in vivo tumor microenvironment, further analysis of patient-derived samples, especially from perinecrotic, oxygen- and nutrient-deprived tumor compartments is of paramount importance for the development of more efficient therapeutic strategies.

5. Conclusions

Our findings suggest that WNT activation plays an important role in promoting the survival of glioblastoma cells under extreme nutrient restrictions. Whether this is limited to malignant glioma-derived cell lines or is a general mechanism in cancer biology requires further attention.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers14133165/s1, Figure S1: (A) glucose starvation enhances the clonogenic capacity in the starved population of cells, (B) *TCF* luciferase activity, and (C) protein expression of active beta-catenin (ABC) in three cell lines; Figure S2: a heat map shows differentially expressed genes (DEGs) within the WNT/β-catenin pathway induced by glucose deprivation; Figure S3: a heat map shows the expression pattern of β-catenin-dependent and independent target genes (retrieved from reference [25]) in our datasets; Figure S4: immunostaining for active β-catenin in glioma cell lines in standard cell culture conditions and under glucose deprivation, (A) immunostaining for active β-catenin in GBM1 and BTSC233 in standard cell culture conditions (450 mg/dL) and under glucose deprivation for 48 h, (B) glucose deprivation enhances *TCF* luciferase activity (WNT activity); Figure S5: in silico drug screen in GBM cell lines with (A) staurosporin, (B) berberine, and (C) LGK974. (D) Other potential chemotherapeutics assessed via robot technology are also shown. Original western blot images from Figure S1C are included.

Author Contributions: Conceptualization, S.Y. and J.M.; methodology, S.Y., P.A., A.-C.N., P.W., N.Q., M.R., D.H., H.L. (Hongjia Liu) and H.L. (Hongde Liu); formal analysis: A.S., H.-J.S., G.R. and S.N.; writing—original draft preparation, S.Y., A.S. and J.M.; supervision, J.M. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

Axin2: axis inhibition protein 2; CNS: central nervous system; CSC: cancer stem-like cells; DMSO: dimethyl sulfoxide; EGF: epidermal growth factor; FGF: fibroblast growth factor; Fwd: forward; GC-MS: gas chromatography mass spectrometry; GLUT1: glucose transporter 1; IDH1: isocitrate dehydrogenase 1; LDH-A: lactate dehydrogenase A; MCT-1:

monocarboxylate transporter 1; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBT: nitro blue tetrazolium chloride; PDH: pyruvate dehydrogenase; PKM2: pyruvate kinase M2; Rev: reverse; rpm: rounds per minute; RT-qPCR: real-time quantitative PCR; TCA: tricarboxylic acid; *TCF/LEF*: T cell factor/lymphoid enhancer factor family; TMZ: temozolomide; WHO: World Health Organization; WNT: wiSuadngless and Int-1; ZEB1: zinc finger E-box binding homeobox 1.

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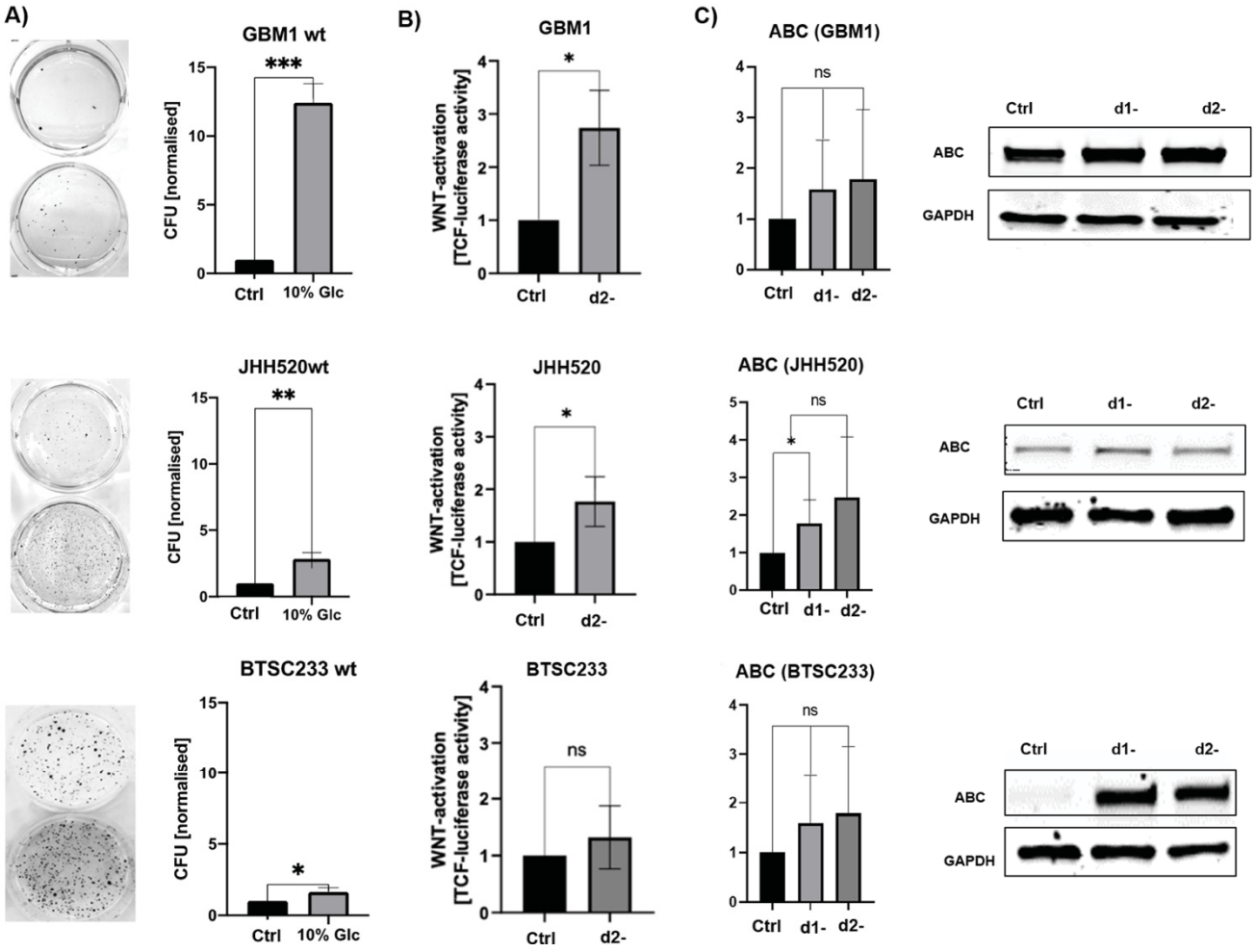
3.2. Supplementary

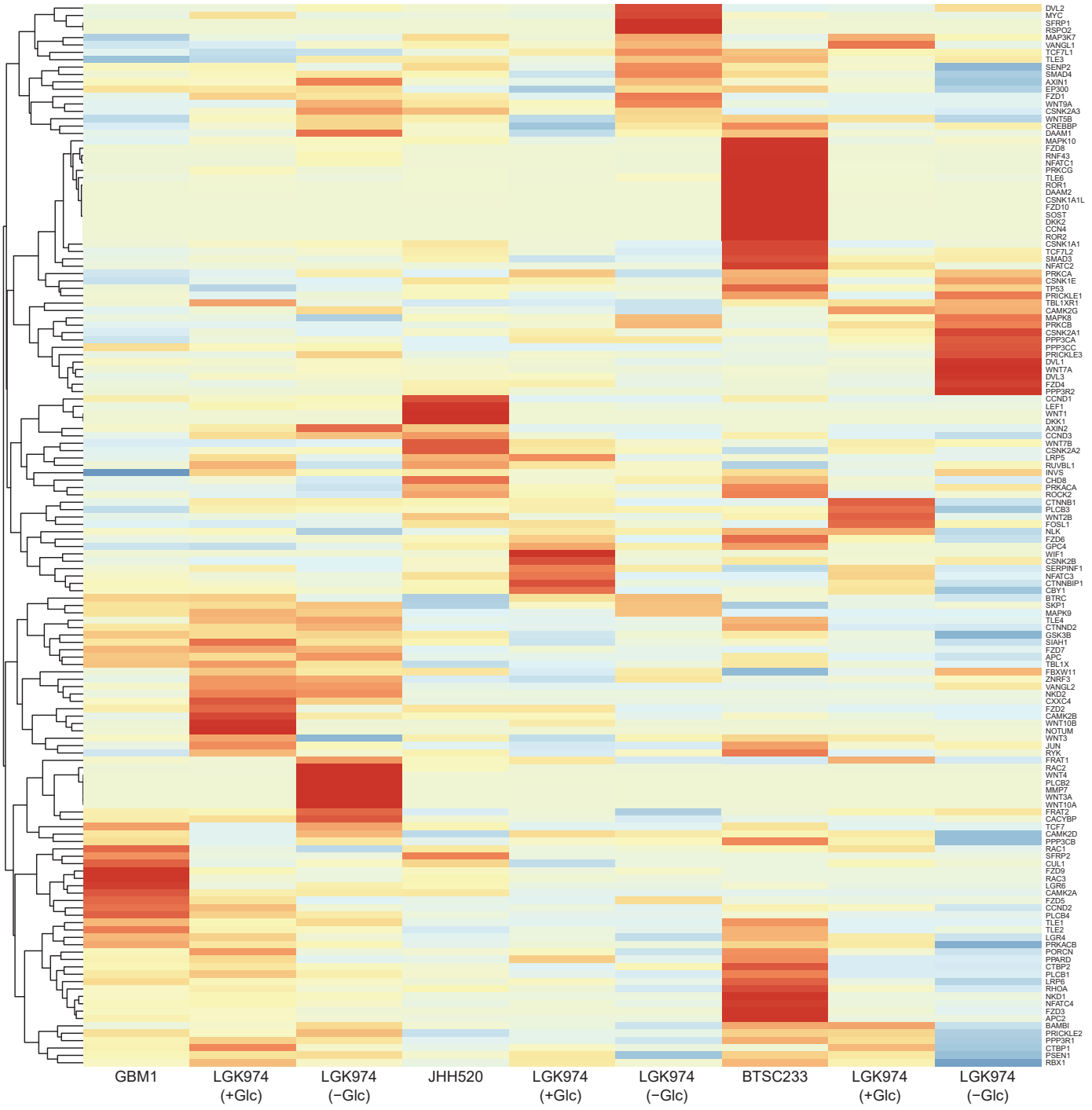
Supplementary figure 1:

A) Glucose starvation enhances the clonogenic capacity in the starved population of cells, B) TCF-luciferase activity and C) protein expression of active β-catenin (ABC) in three cell lines.



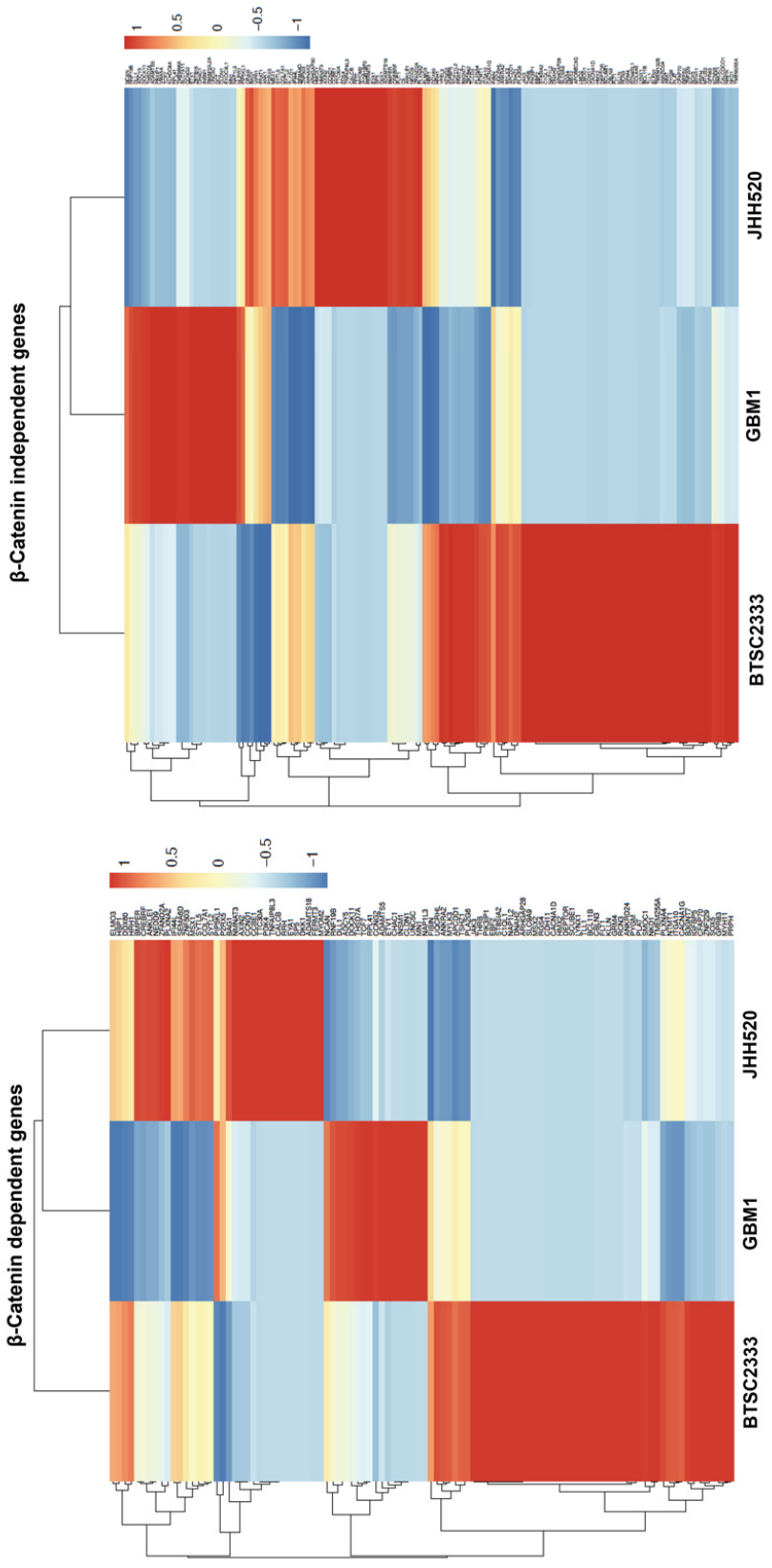
Supplementary figure 2:

A heat map shows differentially expressed genes (DEGs) within the WNT/β-catenin pathway induced by glucose deprivation:



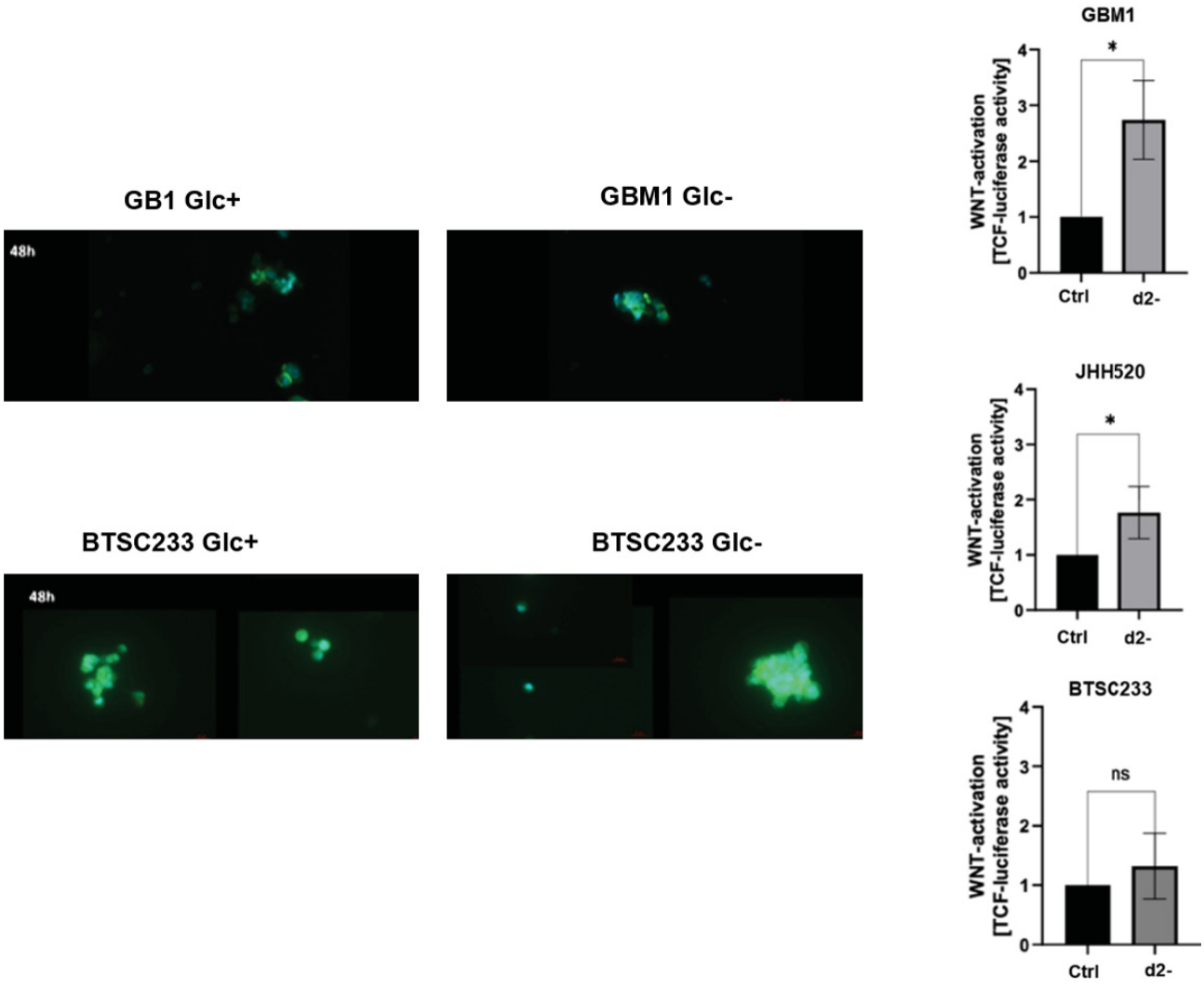
Supplementary figure 3:

A heat map shows the expression pattern of β-catenin-dependent and independent target genes (retrieved from [25]) in our datasets.



Supplementary figure 4.

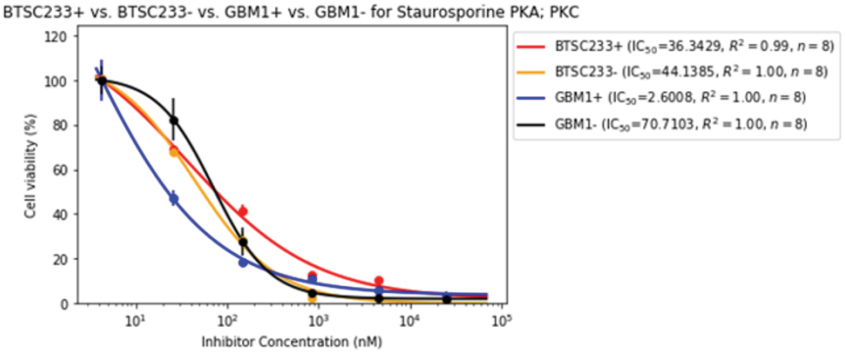
Immunostaining for active β-Catenin in Glioma cell lines in standard cell culture conditions and under glucose deprivation, A) Immunostaining for active β-Catenin in GBM1 and BTSC233 in standard cell culture conditions (450 mg/dL) and under glucose deprivation for 48h, B) Glucose deprivation enhances TCF-luciferase activity (WNT-activity)



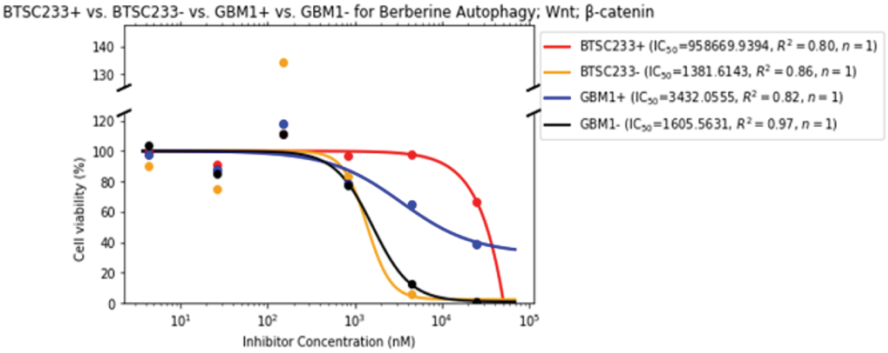
Supplementary figure 5.

In-silico-drug-screen in GBM cell lines with A) Staurosporin, B) Berberine and C) LGK974. D) Other potential chemotherapeutics assessed via robot-technology are also shown.

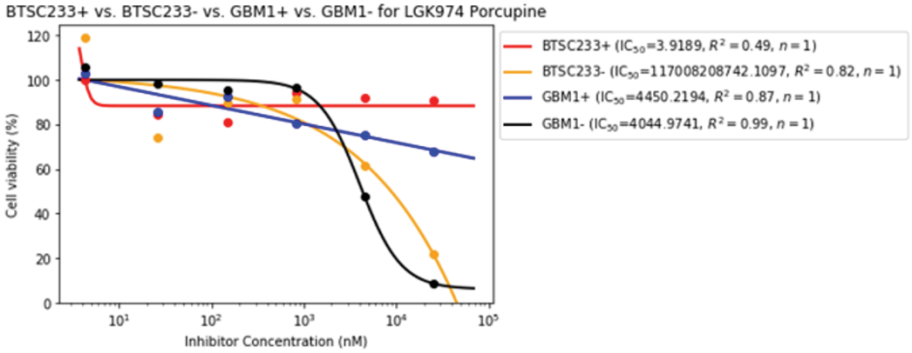
A)



B)



C)



D)

Drugs:	Mechanism:
LGK974	WNT-pathway-inhibitor
Berberine	WNT-pathway-inhibitor
Disulfiram	Aldehyd-deydrogenase-Inhibitor
Andrographolide	NFkB-Inhibitor
Auranofin Bacterial	inhibition of bacterial thioredoxin reductase
Pazopanib	Apoptosis, Autophagy
Entinostat	Apoptosis, Autophagy
Honokiol	Autophagy & Erk-inhibition
Ravoxertinib	Erk-Inhibition
Rigosertibsodium	Apoptosis, PI3K, Polo-like-Kinase
Masitinibmesylate	Apoptosis, c-Kit, FGFR,PDGFR,Scr

4. Discussion

While abnormal WNT activation has been linked to tumorigenesis in many other malignancies [210], its significance in IDH-wildtype glioblastoma remains elusive [321]. In glioblastoma, WNT signaling promotes clonogenicity and proliferation of neural progenitor cells, as well as resistance to alkylating drugs such as temozolomide (TMZ). It has been shown that resveratrol restores the sensitivity of glioma cells to temozolomide treatment through WNT inhibition [322]. WNT activation has also been proposed as a mediator of metabolic resilience in cancer tissue since it mediates various axes contributing to metabolic plasticity [235]. Therefore, the own studies were focused on the WNT-mediated response to glucose deficiency in GSCs. Initially, an MTT test was conducted to determine the proportion of GSCs that survived decreasing or depleted glucose concentrations. A small population of cells was able to survive in a glucose-depleted microenvironment. Decreased glucose levels did not alter the viability of the GSC lines significantly, except in cell line BTSC233, in which a reduced glucose concentration of 45mg/dL significantly lowered viability.

Numerous studies have shown that glucose deprivation predisposes tumor cells in particular to oxidative stress-induced cell death [323]. Tumor cells are dependent on the influx of glucose and become uncoupled from oxidative phosphorylation, which has been described first by Warburg and hence is described as the Warburg effect [236]. One possible explanation is that malignant cells in particular exhibit mitochondria dysfunction. It is possible that in the case of glucose deficiency, oxidative phosphorylation is resorted to, which, however, is associated with increased generation of reactive oxygen species and subsequent apoptosis in the setting of underlying mitochondrial dysfunction [323], [324]. This effect has been demonstrated in glioblastoma cells, but not in astrocytes [325]. The glucose deprivation-induced decreased viability of glioma cells may also be explained by the induction of autophagy to meet endogenous energy requirements [326].

The own investigation of invasive capabilities after glucose deprivation revealed that glucose deprivation resulted in the development of an invasive phenotype, presumably via triggering mesenchymal transition. This adoption of an invasive phenotype in

nutrient-limiting microhabitats has already been demonstrated in other cancer tissues [327]. This invasion-promoting effect was also experimentally recapitulated in glioblastoma cells following glucose deprivation. In particular, the margins of a tumor are particularly predestined for featuring invasive abilities. Interestingly, increased mRNA expression of the unilateral glucose transporter GLUT3, but not GLUT1, was detected at the leading margin of glioblastoma. It was also shown experimentally that GLUT3 correlates with an invasive phenotype. GLUT3 in particular has been correlated with poor clinical outcome and associated with the invasive phenotype of gliomas [328]. Both *GLUT1* and *GLUT3* are known to be WNT/β-catenin target genes [199], [329] and GLUT3-overexpressing glioblastoma cells also show increased expression of CD44 transcripts, suggesting a link to the WNT/β-catenin pathway [328]. Conversely, hyperglycemia has also been linked to the development of an invasive phenotype in glioblastoma cells, suggesting that decreased glucose concentration or glucose deprivation is not the only trigger for the development of an invasive phenotype [330].

The soft agar colony formation experiments was included in the own studies to quantify self-renewal capabilities as a proxy of stem-like cell phenotype, and it was found that GSCs subjected to decreased glucose concentration for four weeks exhibited higher clonogenic capacity. This suggests that nutritional restriction enriches for GSCs. Glucose restriction has been linked to an increased stem-like phenotype in cancer before [331]. Stem-like glioma cells are characterized by increased resistance to radiochemotherapy and are considered a particularly severe therapeutic challenge [332]. In radiochemotherapy-resistant glioblastoma stem-like cells, metabolic adaptation to reduced glucose concentrations has also been demonstrated [333]. Stemness is essential for promoting the longevity, self-renewal, and metabolic adaptability of a tumor bulk. A number of stemness factors and phylogenetically conserved stemness pathways, including the WNT pathway, have been implicated as drivers of cancer cell stemness and tumor formation [334].

The own study focused on WNT as a possible driver of metabolic plasticity. Glucose deprivation affected both the local β-catenin and β-catenin target gene transcription, as well as global gene transcription. Furthermore, the significant increase in luciferase signaling revealed that the canonical WNT pathway was activated in GSCs in response

to glucose deprivation. Based on these findings, one can reason that in response to metabolic stress, glucose-depleted GSCs activate canonical WNT signaling.
In addition, numerous nutritive stress stimuli can induce reprogramming by the PTEN, WNT and Hedgehog pathways in glioblastoma cell lines [10]. A number of β-catenin-mediated mechanisms have been described to confer resilience of tumor cells to glucose deprivation, for example, via oncogenic signaling pathways involving PIK3CA- and EGFR-mediated activation of β-catenin [335]. Also, WNT/β-catenin-mediated resilience via macrophage-secreted interleukin 35 (IL-35) has been reported, which illustrates the complexity of metabolic reprogramming via WNT activation [336]. In contrast, there are also studies indicating that glucose deprivation may mediate proteasomal degradation of β-catenin via protein kinase C [337].

Using an in-silico drug screen, it was shown that glucose deprivation sensitized GSCs to certain drugs such as disulfiram (an aldehyde dehydrogenase inhibitor), andrographolide (a nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFkB) inhibitor), pazopanib (tyrosine kinase inhibitor), entinostat (a histone deacetylases (HDAC) inhibitor), honokiol (an extracellular signal-regulated kinase (ERK) inhibitor), ravoxertinib (an ERK inhibitor), rigosertibsodium (a Polo-like kinase-1 (PLK1)inhibitor), and masitinbmesylate (a tyrosine kinase inhibitor). Interestingly, increased chemotherapy sensitivity under glucose deprivation was revealed for two WNT inhibitors: LGK974 and berberine. WNT inhibition with the porcupine-inhibitor LGK974, which disrupts the endoplasmatic release of WNT [338], resulted in significantly lower survival under glucose deprivation in a dose-dependent manner. Although this outcome is most likely due to direct WNT/β-catenin suppression, off-target effects of LGK974 cannot be ruled out. Besides this, the heterogenic transcriptional traits of the GSCs used in this study (BTSC233: IDH-wildtype glioblastoma, mesenchymal subtype; JHH520: IDH-wildtype mesenchymal subtype; GBM1: IDH-wildtype, classic subtype) might be a significant factor for the minor variations in the observed responses to metabolic stress. It has been previously explored how such inherent variability between cancer cell lines causes experimental inconsistencies.

LGK974 has been found in several studies to significantly reduce the viability, proliferation, and clonogenicity of glioblastoma cells [273], [339]. In particular, the reduction of the self-renewing population is of considerable importance, as such stem

cell-like capabilities may foster the acquisition of high metabolic resilience, which is a particular challenge in the development of efficient therapeutic strategies [340]. It has been shown that LGK974 impairs mitochondrial function, which is a significant systemic off-target and adverse effect [341]. However, the above-mentioned reduced metabolic resilience induced by LGK974 treatment is likely due to the inhibition of WNT/β-catenin-driven metabolic reprogramming.

The use of GC-MS enabled to define the metabolic profile of GSCs under glucose deprivation, WNT inhibition, and a combination of both. These experiments revealed significant alterations in a number of metabolites, both when WNT inhibition was used alone and when it was combined with glucose depletion. In contrast to other glucogenic amino acids (such as glutamate, 5-oxoproline, methionine, and aspartate), alanine was considerably reduced in starved cell lines, most likely as a result of anaplerosis. Further research is necessary to determine whether these effects upon glucose withdrawal are unique to the metabolic milieu of gliomas or also prevalent in other malignant cell types. The non-polar, non-essential, and glucogenic amino acid has been highlighted several times in the literature in the context of amino acid metabolism of glioblastoma cell lines. As previously detailed, the Warburg effect is exploited in neoplastic cells instead of oxidative phosphorylation [236]. The accumulated pyruvate is converted to alanine by glutamate pyruvate transaminase and can be utilized for numerous metabolic or biosynthetic pathways. For example, the accumulated pyruvate is further metabolized to glutamine and thus used for glutaminolysis. Pyruvate, lactate, alanine, and glutamine are also gluconeogenic precursors and can be incorporated into gluconeogenesis [342]. It was demonstrated by GC-MS that isotopically labeled [3-13C]alanine was converted to lactate via pyruvate in glioblastoma cells. The isotopically labeled pyruvate was used for anaplerosis of TCA and numerous TCA intermediates were obtained that also carried the isotopically labeled carbon, which can ultimately be fed to biosynthesis [343]. Thus, this amino acid plays a crucial role in driving energy metabolism in glioblastoma cells. Therefore, it makes sense that especially during glucose deprivation, alanine is reduced from all glucogenic amino acids and hence should be exploited first and foremost.

Remarkably, during combined WNT inhibition and glucose restriction, the levels of the ketogenic amino acids lysine and leucine were lowered in all cell lines. This suggests that WNT signaling has yet another function in the previously reported process of lipid

metabolism. The own findings contribute to a more thorough knowledge of WNT signaling as a key participant in the regulation of ketogenesis through both the use of ketogenic amino acids and the beta-oxidation of fatty acids. Beyond beta-oxidation, WNT regulates ketogenesis via the transcription of key ketogenesis enzymes such as HMGCS2 [344]. Especially in central nervous tissues, the provision of ketone bodies is an indispensable alternative energy source to glucose. Hepatocytes usually produce the main proportion of ketone bodies which are transported across the brain-blood barrier via monocarboxylate transporters and can be utilized for energy production instead of glucose [345], [346]. In addition to the beta-oxidation of fatty acids, ketone bodies can also be obtained alternatively by utilizing ketogenic amino acids. Strictly ketogenic amino acids are the essential amino acids lysine and leucine. Various metabolic pathways can be driven via acetyl-coA, such as the anaplerosis of TCA [347]. Ketogenic diets have already been proposed as a possible therapeutic approach that may act in addition to the (pharmacological) glucose deprivation taking place in glioblastoma cells. This is because it has been experimentally demonstrated in glioblastoma cells that a ketogenic diet does attenuate stemness and proliferation [348]. WNT/β-catenin-mediated fatty acid oxidation and WNT/β-catenin transcriptional-mediated ketogenesis have been described more frequently [349]. The own findings contribute to the previous understanding of WNT/β-catenin-mediated ketogenesis by fatty acid oxidation. They show that the limited availability of ketogenic amino acids for subsequent usage in anaplerotic processes in the tricarboxylic acid cycle may be the cause for the reduced survival of glucose-deprived GSCs following concurrent WNT inhibition. In addition, alpha-ketoglutarate, citrate and isocitrate, malate, and fumarate were reduced in starving GSCs irrespective of WNT inhibition, suggesting a general mechanism, such as fast integration into the reaction chains of the tricarboxylic acid cycle.

Furthermore, hydroxyglutarate, myo-inositol, succinate, and fumarate were among the oncometabolites that were impacted by WNT suppression of glucose-deprived GSCs. In IDH-mutant glioma cells, 2-hydroxyglutarate accumulates in the tumor cells as the mutant IDH enzymes generate 2-hydroxyglutarate instead of alpha-ketoglutarate. Consequently, due to the structural similarity to alpha-ketoglutarate, alpha-ketoglutarate-dependent dioxygenases are competitively inhibited by 2-hydroxyglutarate, thus promoting epigenetic rerogramming, HIF1α stabilization and ultimately leading to tumorigenesis [350]. Nevertheless, 2-hydroxyglutarate can also be formed and

accumulated in IDH-wildtype glioma cells [351]. The WNT pathway may have an impact on the formation of cancers that are oncometabolically driven, as the finding that 2-hydroxyglutarate was substantially decreased in IDH-wildtype glioblastoma cells following treatment with LGK974. The phosphoethanolamine level was also reduced by WNT inhibition, while glucose deprivation led to its increase. Recent research revealed that IDH-mutant gliomas downregulate phosphocholine and phosphoethanolamine production in a 2-hydroxyglutarate-dependent way [352]. Thus, the reduced quantity of phosphoethanolamines may also be explained by the pharmacological inhibition of WNT and the associated reduced expression of 2-hydroxyglutarate. Phosphoethanolamine is an important component of phospholipids and thus essential for incorporation into cell membranes and indispensable for cell division. Phosphoethanolamine expression has been correlated with the tumorigenicity of glioblastoma cells and its accumulation also protects tumor cells against nutritive stress, initially probably against glutamine starvation [353].

It has been previously discussed that the primary mechanism involving protein kinase C (PKC) is what causes β-catenin to degrade in response to glucose deprivation [337]. Interestingly, glucose deprivation activates AMP-activated protein kinase (AMPK), which enables β-catenin-mediated gene transcription via phosphorylation and nuclear exportation of histone deacetylase 5 (HDAC5). The precise mechanism by which glucose deprivation increases β-catenin activity is yet elusive, however, WNT activation played a critical role in preserving cell viability in GSCs under severe nutrient restriction.

In conclusion, the research results summarized in this thesis unveil unexpected pleiotropic effects conferring WNT-mediated metabolic plasticity and resilience in GSC lines. In addition, these findings suggest that metabolic fingerprints may complement traditional molecular biomarkers to improve prediction of tumor behavior and clinical prognosis. Further examination of *in vivo* glioblastoma models and patient-derived tissue samples, particularly from perinecrotic, oxygen- and nutrient-deprived tumor compartments, is crucial for the development of more effective therapeutic approaches targeting GSCs because *in vitro* tumor models may not accurately mirror the *in vivo* tumor microenvironment.

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6. List of Figures

All illustrations of the dissertation (introduction & discussion) were designed and conceptualized by myself employing partial graphic elements from ***Servier Medical Art***, licensed under a Create Commons Attribution 3.0 unported license.

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