REVIEW PAPER

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Contribution of the Wnt Pathway to Defining Biology of Glioblastoma

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Abstract

Glioblastoma (GBM), a highly lethal brain tumor, has been comprehensively characterized at the molecular level with the identification of several potential treatment targets. Data concerning the Wnt pathway are relatively sparse, but apparently very important in defining several aspects of tumor biology. The Wnt ligands are involved in numerous basic biological processes including regulation of embryogenic development, cell fate determination, and organogenesis, but growing amount of data also support the roles of Wnt pathways in the formation of many tumors, including gliomas. Two main Wnt pathways are distinguished: the canonical (β-catenin) and non-canonical (planar cell polarity, Wnt/Ca^{2+}) routes. Wnt signaling regulates glioma stem cells (GSCs), thereby defining invasive potential, recurrence, and treatment resistance of GBM. Some observations suggest that the Wnt pathways are differentially active in molecular subtypes of this tumor, thereby may also guide prognostication and novel therapeutic decisions. In this review, we highlight main elements and biological relevance of the Wnt pathways, primarily focusing on the pathogenesis and subtypes of GBM. Finally, we briefly summarize newer therapeutic strategies targeting networks of the Wnt signaling cascades and their molecular associates that appear to be marked contributors to GBM aggressiveness.

Keywords Glioblastoma · Molecular subgroups · Wnt pathways · Glioma stem cells · Treatment targets

Introduction

Glioblastoma (GBM) is the most common and most aggressive glioma, defined as grade IV according to the World Health Organization (WHO) classification. Primary GBMs (90%) arise *de novo*, while secondary GBMs (10%) are formed by transformation from lower-grade gliomas. Primary GBM has a poor prognosis with around 20 months of median survival time under current standard of care (Weller et al. 2017; Desjardins et al. 2018), and typically develops in older patients with a median age of 62 years. Secondary GBM occurs in younger patients with a median age of 45 years, and has better clinical outcome with longer, 31 months of median overall survival time (Ohgaki and Kleihues 2012; Mansouri et al. 2017).

The current standard of care involves surgical resection of the tumor, radiotherapy, and temozolomide chemotherapy often supplemented by other modalities such as bevacizumab, an anti-VEGF (vascular endothelial cell growth factor) monoclonal antibody (Stupp et al. 2005; Shi et al. 2018). Due to the invasiveness of the tumor, an accurate determination of the tumor edges as well as the spread of malignant cells is difficult during both surgery and irradiation, which greatly contributes to tumor recurrence. Apart from a small number of cases, the tumor returns in a year, most commonly within 2 cm of the original lesion site (Gaspar et al. 1992). Until recently, the histological work up served as an ultimate tool to define diagnosis, and to reflect a great degree of inter- and intratumor heterogeneity of the GBM. In 2008, The Cancer Genome Atlas Network (TCGA) reported the most significant somatic molecular alterations including single nucleotide polymorphisms (SNPs), copy number variations (CNVs), and chromosomal structural variations genome-wide in GBMs (The Cancer Genome Atlas Network 2008). Two years later, integrated analyses

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of genomic and transcriptomic data showed a separation of molecular GBM subtypes named "classical," "mesenchymal," "proneural," and "neural" (Verhaak et al. 2010). Subsequently, the latter subgroup was abandoned due to a likely contamination of normal cells in the originally identified neural sample subset (Wang et al. 2017). In the meantime, extensive OMICS profiling revealed that molecular characteristics not only associate with glioma subtypes but may even occasionally over-ride histological grade. Therefore, a group of experts generated a consensus statement for the integration of molecular and histological information, and published a revision of the WHO classification for brain tumors in 2016 (Louis et al. 2016). Accordingly, the first level of discrimination includes the mutational status of isocitrate dehydrogenase (*IDH*) genes for both low- and high-grade gliomas. Focusing here only on high-grade gliomas, patients with *IDH* mutant GBM tend to be younger and have somewhat better prognosis than those with *IDH* wild-type (Verhaak et al. 2010; Nagy et al. 2017). The *IDH* mutant GBMs belong to the proneural molecular subtype that is also characterized by the glioma CpG island methylation pattern (G-CIMP) (Noushmehr et al. 2010; Verhaak et al. 2010). The *IDH* mutational and G-CIMP status are not independent of each other, as the defective IDH enzymes produce an oncometabolite D-2-hydroxyglutarate exerting significant epigenetic regulatory activities (Noushmehr et al. 2010; Kalovits et al. 2018).

Several key signaling pathways (including the RTK/PI3K, p53, and RB pathways) have been identified in the development of GBMs (Verhaak et al. 2010), among which the Wnt pathway, related to stem cell maintenance and differentiation, may have a particular importance. GSCs greatly contribute to tumor development, recurrence, and progression (Sandberg et al. 2013). The expression patterns of target genes controlled by Wnt, Hedgehog, and Notch pathway signaling molecules are altered in cancer stem cells (CSCs) including GSCs (Lai et al. 2003). Dysregulation in Wnt pathways plays a pivotal role in GSC biology. Our preliminary observations also suggest that activation of these pathways is not independent of the GBM molecular subtypes. Therefore, in the following overview, we will summarize the roles of CSCs and GSCs in gliomagenesis, and highlight the importance of Wnt pathways in these processes in GBM and its molecular subtypes.

CSC and GSC

Initially generated a century ago, the CSC theory of cancer development postulates that tumor cells originate from precursor cells and evolve through multiple clonal hierarchies (Clarke et al. 2006; Dalerba et al. 2007). CSCs are basically stem cells with cancer characteristics, which are capable of self-renewal and differentiation into multiple cell types, thereby contributing to phenotypic and molecular heterogeneity of tumors (Roarty and Rosen 2010). Intratumor heterogeneity results from clonal heterogeneity of cells evolving through a stochastic (or clonal evolution) or a hierarchy (or CSC) model (see details of these models in Rich 2016), and is reflected by genetic, epigenetic, and histological markers (Ellis H.P et al. 2015). In gliomas, CSCs are called GSCs or glioma-initiating cells (GICs) that share similarities with neural stem cells, e.g., by expressing CD133, CD44, and Nestin markers (Lee et al. 2006; Petropoulos et al. 2018). GSCs may evolve through multiple mechanisms and from more than one cell type (i.e., mutated neural stem cells, mutated neural precursor cells, and glial precursors such as oligodendrocyte precursor cells) (Jackson et al. 2014; Lee et al. 2018). Dedifferentiation of differentiated cells, however, is also a mechanism to generate CSCs (Kim et al. 2017). An elevated CSC pool can be observed in GBMs and other malignant brain tumors, which are formed by increased symmetric stem cell division or differentiated cell reprogramming. These mechanisms greatly contribute to the tumor's phenotypic plasticity (Gao et al. 2014; Safa et al. 2015). In CSCs and GSCs, the activity of Wnt pathway molecules increases as compared to that in normal stem cells (Skoda et al. 2016). *Vice versa*, inhibition of Wnt pathway molecules reduces CSC stemness (Kim et al. 2017). $CD133^+$ GSCs are more resistant to irradiation than the $CD133^-$ cells due to more effective DNA repair mechanisms in the positive than in the negative cells (Bao et al. 2006).

Available data suggest that GSCs are the main promoters of GBM invasion and tumor recurrence (Xie et al. 2014). CSCs are also important drivers of metastasis formation in case of other solid tumors. These stem cells evade immune responses, develop dormancy, and create a special microenvironment. However, the converted microenvironment also feeds back to maintaining, controlling, and regulating the CSC and GSC pools (Safa et al. 2015). As a result of chemotherapy, irradiation, or hypoxia, the GSC population within the tumor becomes active with distinct gene and protein expression profiles (e.g., altered expression of P-glycoprotein, mammalian target of rapamycin (mTOR) and CD133, or unmethylation of DNA repair genes) to protect the tumor from apoptosis and treatment interventions (Xie et al. 2014; Safa et al. 2015). Furthermore, these stem cells render the tumor resistant against DNA fragmentation, cell cycle inhibitory factors, and mechanisms inhibiting cytoskeletal or microtubule formation (Yeung et al. 2010; Ogawa et al. 2013). A small group of CSCs is always retained within established tumors and underlies invasive processes or initiates recurrence after therapy (Gil et al. 2008). Altogether, evidence strongly supports that CSCs in solid tumors and GSCs in gliomas play prominent roles in progression, invasion, and recurrence.

GBM Molecular Heterogeneity as Defined in Clinical Practice

During evolution of gliomas, somatic mutations accumulate in the tumor cells. These include mutations of the *IDH* genes present in 70% of low-grade gliomas (II–III) and 7–10% of high-grade gliomas (IV). Of the five isotypes, IDH-1 is a NADP + -dependent enzyme expressed in the cytoplasm, endoplasmic reticulum, and peroxisomes. In gliomas, the mutation of *IDH-1* most commonly (in 90%) affects the arginine residue at codon 132 (IDH-1 R132H) (Parsons et al. 2008; Ma et al. 2015). IDH-2 is also a NADP +- dependent enzyme, but localized in mitochondria, and most frequently its arginine residues are affected at codons 140 (R140) and 172 (R172). These mutations result in a significant reduction in the enzymatic activity of IDH-1 and − 2. IDH-3, IDH-4, and IDH-5 are NAD + -dependent isoenzymes in mitochondria, but lack characteristic mutations in gliomas (Kalovits et al. 2018). Wild-type IDH-1 and IDH-2 catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate (αKG), while simultaneously reduce NADP + to NADPH + H. The efficiency of the mutated enzymes to convert isocitrate to αKG as well as to generate NADPH is decreased (loss of function), while there is an increase in the formation of D-2-hydroxyglutarate (D2HG) oncometabolite along with NADP + from αKG and NADPH (gain of new function) (Fig. 1). The concentration of the toxic D2HG is very low in normal cells, as D2HG dehydrogenase (D2HGDH) converts it back to αKG. In contrast, the D2HG concentration greatly rises with multiple biological consequences, while the decreased availability of NADPH also profoundly weakens the antioxidant and detoxifying capacities in IDH-1 and IDH-2 mutated cells (Kalovits et al. 2018). Most recently, epigenetic effects of D2HG have been explored since D2HG competes for the αKG-binding sites and inhibits the αKG-dependent dioxygenases, most importantly the JmjC domain-containing histone demethylases and the TET (ten-eleven translocation) family of DNA hydroxylases. An important net effect is hypermethylation of CpG islands genome-wide (Figueroa et al. 2010; Kalovits et al. 2018) reflected by the G-CIMP profile in the *IDH-1* mutated proneural subtype of GBM (Noushmehr et al. 2010). Because of its marked discriminative effects in tumor biology and prognosis, determination of the IDH status has become part of clinical histological evaluations in gliomas including GBM (Louis et al. 2016). Although not closely relevant to the present survey, we mention here in parenthesis that identification of the other two molecular GBM subtypes (classical and mesenchymal) is also feasible in the clinical setting based on our recent studies (Nagy et al. 2017).
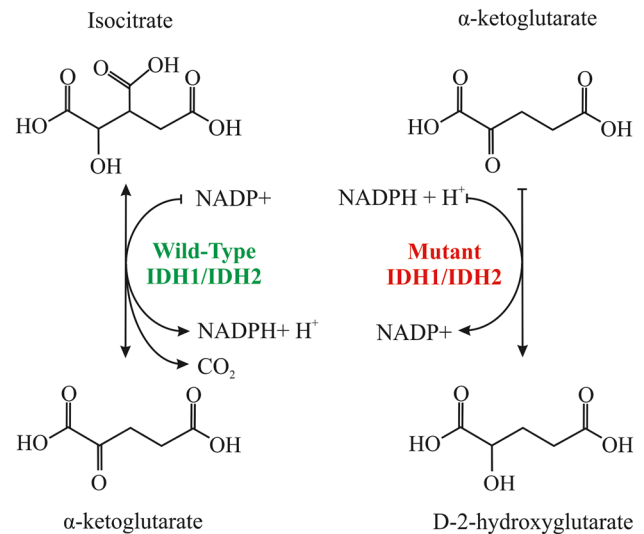

Fig. 1 Catalytic functions of the wild-type and mutated IDH1/IDH2 enzymes. In normal cells, the wild-type IDH1/IDH2 converts isocitrate to α-ketoglutarate while it reduces NADP + to NADPH + H (a reversible reaction). In cancer cells, the mutant IDH1/IDH2 generates D-2-hydroxyglutarate (D2HG) oncometabolite from α-ketoglutarate while it consumes NADPH (an irreversible reaction)

The Wnt Ligand Family

The *Wnt* gene in mammals was first described in 1982 (Nusse and Varmus 1982). Originally, it was called Integration 1 (*int1*) and identified in the mouse mammary tumor virus (MMTV)—induced breast cancer (Nusse and Varmus 1982). Six years before the description of *int1*, the wingless (*wg*) gene was discovered in Drosophila melanogaster, and named so, because due to its mutation, the animal did not develop wings (Sharma and Chopra 1976). In 1987, *int1* was recognized as the mammalian equivalent of the Drosophila *wg* gene. The mammalian Wingless-related integration site (*Wnt*) gene was generated by the combination of Wingless and Integration 1 (Nusse et al. 1991). There are now at least 19 known Wnt ligands with more than 15 receptors and co-receptors that can be divided into seven protein families (Willert and Nusse 2012). Wnt molecules are cysteine-rich, 350–400 amino acid long, secreted glycoproteins (Kikuchi et al. 2011). Two major Wnt routes are distinguished: the canonical and the non-canonical signaling pathways. Both the canonical and the non-canonical pathways are highly conserved, and essential in the early stages of embryonic development, formation of body axis, cell fate determination, and in the definition of cell migration and proliferation potential (Peifer and Polakis 2000; Tada et al. 2002). Hence Wnt signaling plays important roles in a multitude of basic biological processes, but is also involved in the development of neurodegenerative diseases and cancers (Nayak et al. 2016). Below, we briefly describe the two Wnt signaling

pathways, while for further details of this topic refer the readers to a comprehensive review (Kahn 2014).

The β-Catenin-Dependent or "Canonical" Wnt Pathway

Main elements of this pathway are composed of the Frizzled (FZD) receptors along with their co-receptors, the low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6), the dishevelled segment polarity protein 1 (DVL1), and the axis inhibition protein (AXIN) (MacDonald et al. 2009; Baarsma et al. 2013). AXIN functions as a scaffold protein.

Without binding of a Wnt ligand to its receptor, β-catenin is marked for degradation by casein kinase 1 (CK1)- and glycogen synthase kinase 3β (GSK-3β)-mediated phosphorylation and β-transducin repeating protein (β-TrCP)-mediated ubiquitination in a cytoplasmic complex, causing a low expression of Wnt signaling marker genes (Fig. 2) (Komiya and Habas 2008; Santiago et al. 2017). Low β-catenin concentration in the nucleus causes a transcriptional co-repressor, Groucho, to bind to the lymphoid enhancer factor 1 (LEF1) and T-cell factor 4 (TCF4), and thereby negatively regulating the expression of Wnt signaling marker genes (Fig. 2) (Steinke and Xue 2014).

Upon binding of a Wnt ligand to its receptor FZD and co-receptors LRP 5/6, their intracellular components will
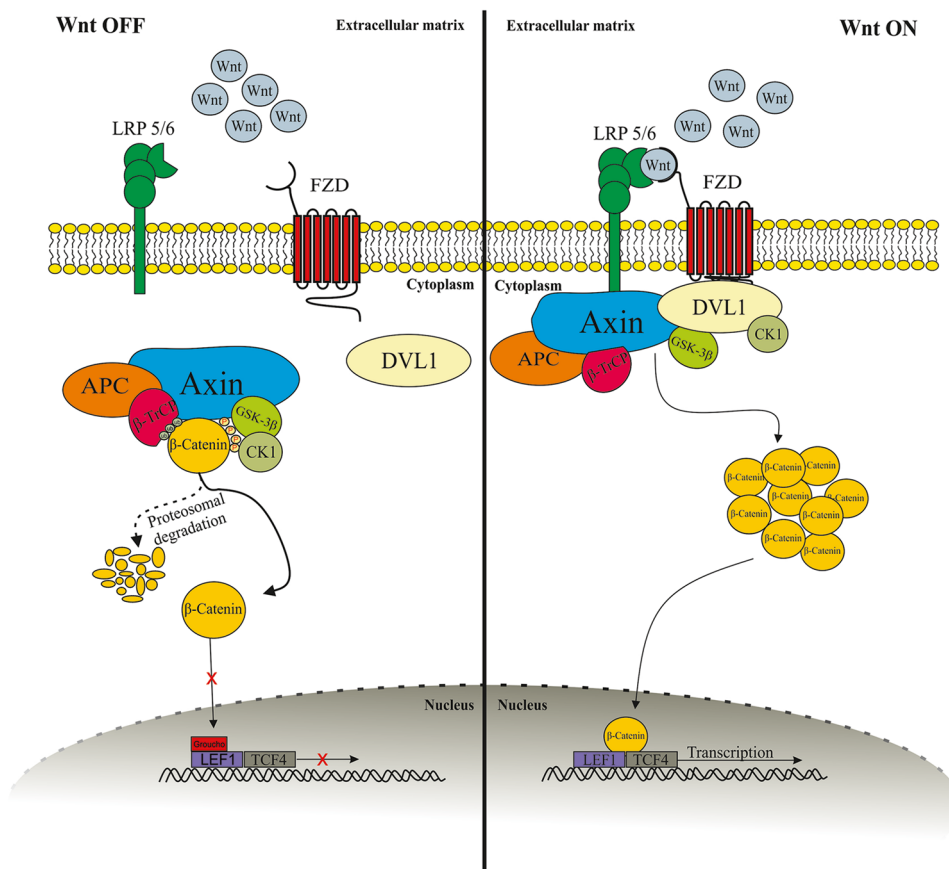

Fig. 2 β-catenin-dependent or "canonical" Wnt pathway. When the Wnt ligand does not bind to its FZD receptor, β-catenin is marked for degradation. In this scenario, AXIN, a scaffold protein, is associated with β-catenin, APC, and two serine–threonine kinases, CK1 and GSK-3β, which together form in a cytoplasmic complex. In the absence of Wnt ligands, β-catenin is retained in the complex and marked for proteosomal degradation by CK1 and GSK-3β-mediated phosphorylation, and β-TrCP-mediated ubiquitination. Low β-catenin level in the nucleus promotes Groucho, a transcriptional co-repressor, to bind to LEF1 and TCF4, and thereby negatively regulating the expression of Wnt target genes. When a Wnt ligand binds to its receptor FZD and co-receptor LRP 5/6, their intracellular components will undergo phosphorylation, causing AXIN to bind to LRP 5/6 and DVL1 to FZD. AXIN is unstable in a dephosphorylated state and its level drops. Meanwhile, DVL1 gets activated by phosphorylation and inhibits the activity of GSK-3β and CK1. Consequently, the protein complex cannot phosphorylate and ubiquitinate β-catenin. The cytoplasmic level of free β-catenin increases and the molecule translocates to the nucleus where it forms a transcription complex with LEF1 and TCF4. The complex induces the expression of Wnt target genes, such as *c-myc* and *CYCD1* that regulate cell proliferation and differentiation as well as increases the levels of MMPs that promote matrix degradation and tumor invasion (Komiya and Habas 2008; MacDonald et al. 2009; Kahn 2014)

undergo phosphorylation, causing inactivation of the cytoplasmic complex (Santiago et al. 2017). Consequently, the cytoplasmic level of free β-catenin increases, and the molecule translocates to the nucleus. There, β-catenin forms a transcription complex with LEF1 and TCF4 (Fig. 2). The complex induces the expression of Wnt target genes, such as *c-myc* and Cyclin D1 (*CYCD1*) that regulate cell proliferation and differentiation. The complex also increases the level of matrix metalloproteinases (MMP), key molecules in matrix degradation, tumor invasion, and infiltration (Shu et al. 2005; Klaus and Birchmeier 2008; Valenta et al. 2012).

The best characterized ligands for the canonical pathway are Wnt1, Wnt3a, and Wnt7a, and the typical receptors are FZD1, FZD4, and FZD9.

The β-Catenin-Independent or "Non-canonical" Wnt Pathway

This signal path can be divided into two sub-pathways called the planar cell polarity (PCP) and the Wnt/Ca^{2+} cascade (Fig. 3) (Gordon and Nusse 2006).

The PCP pathway determines the apical and basolateral polarity of cells (Darken et al. 2002). As a major regulator of the cytoskeletal actin, the PCP pathway is important in defining cellular shape and migration, while also organizes intracellular organelles (Komiya and Habas 2008). Upon binding of a Wnt protein to its receptor FZD and co-receptor, receptor-like tyrosine kinase (Ryk) or receptor tyrosine kinase-like orphan receptor (ROR), activation of the dishevelled-associated activator of morphogenesis 1 (DAAM1), Profilin and the Rho- and Ras-related C3 botulinum toxin substrate 1 (RAC1) protein ensues through DVL1. DAAM1 activates RhoA and regulates the cytoskeleton through the Rho-associated kinase (ROCK). RAC1 activates the c-Jun terminal kinases (JNKs) that induce directly, or through the activator protein-1 (AP-1) family indirectly, cytoskeletal changes (Komiya and Habas 2008).

The Wnt/Ca^{2+} cascade has a major impact on the formation of embryonic dorsal axis, and on the determination of cell fate, gastrulation, and tissue genesis. When the Wnt ligand binds to its receptor FZD, DVL1 will get activated with the help of a G-protein. Phospholipase C (PLC) elevates the intracellular Ca^{2+} concentration. The elevated
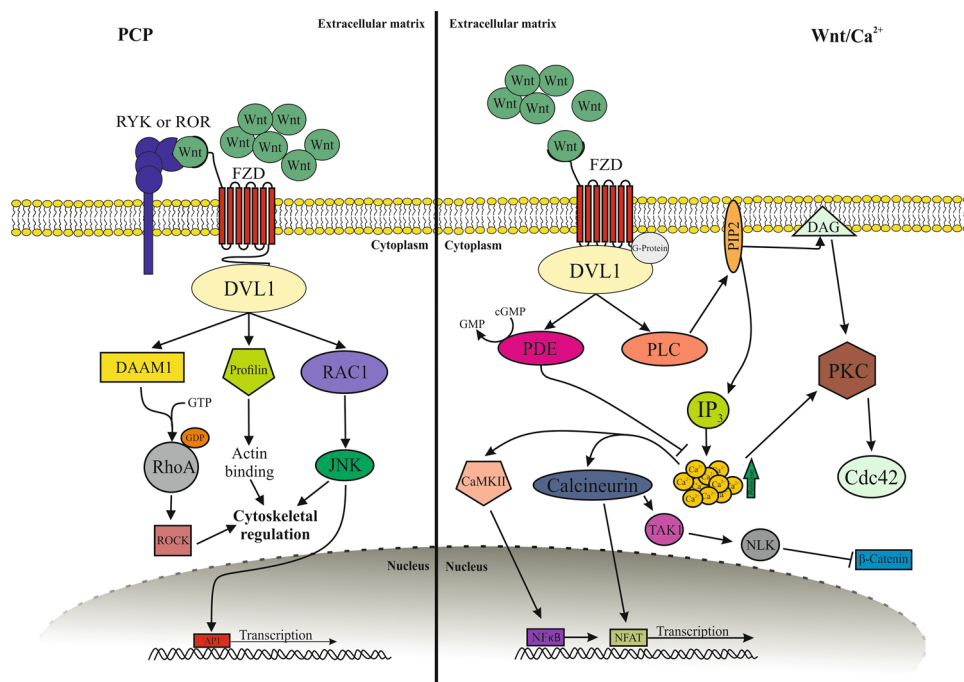

Fig. 3 β-catenin-independent or "non-canonical" Wnt pathway. PCP: Engagement of a Wnt protein with its receptor (FZD) and co-receptor (Ryk or ROR) causes activation of the DAAM1, Profilin, and the RAC1 protein through DVL1. DAAM1 activates RhoA and regulates the cytoskeleton through ROCK. RAC1 activates JNKs that induce directly, or through the AP-1 family indirectly, cytoskeletal changes. Wnt/Ca2+: Engagement of a Wnt ligand with its FZD receptor, DVL1 activates the cGMP-specific PDE and PLC. The active PLC cleaves the membrane-bound PIP2 into DAG and IP³. IP³ releases

Ca^{2+} from the endoplasmic reticulum to the cytoplasm. The increasing Ca^{2+} concentration and the activated DAG trigger activation of PKC that in turn activates the Cdc42 protein. The elevated Ca^{2+} concentration also triggers CaMKII regulating the activity of the NFκB transcription factor, and calcineurin raising the activity of the NFAT transcription factor. On the contrary, however, binding of certain Wnt ligands to FZD may switch calcineurin to inhibit β-catenin and the canonical pathway by activated kinases, TAK1 and NLK (Komiya and Habas 2008; Kahn 2014)

level of Ca^{2+} leads to the activation of two Ca^{2+}-dependent proteins Ca^{2+}/calmodulin-dependent protein kinase II (CaM-KII) and calcineurin and two transcription factors, nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) and nuclear factor of activated T cell (NFAT) that are important for cell survival. However, certain types of Wnt ligands may switch the function of calcineurin to inhibit β-catenin and the canonical pathway, by the transforming growth factor beta-activated kinase (TAK1) and nemo-like kinase (NLK) (Fig. 3) (Komiya and Habas 2008).

The malfunction of non-canonical pathway signaling is often caused by gene mutations observed in neuronal closure defects or ciliopathies. However, an overactivity of this pathway may also be observed in cancer (e.g., breast and ovarian cancer) and advanced melanoma (Daulat and Borg 2017).

The most characteristic ligands for the non-canonical pathway are Wnt4, Wnt5a, Wnt7b, and Wnt11, and most typical receptors are FZD2, FZD3, FZD6, and FZD7.

The Role of Wnt Signaling in Oncogenesis

The Wnt pathway is known to have cross-talk with other important cell signaling pathways including the Notch, Hedgehog, and the epithelial growth factor receptor (EGFR) signaling cascades (Suwala et al. 2016).

Mutations in molecules that are involved in the Wnt signaling cascades play a decisive role in the pathogenesis of several tumors. For example, mutations of the APC molecule in 80% and of β-catenin in 10% are responsible for an aberrant activation of the Wnt pathway in colorectal carcinomas. Consequently, the protein complex is unable to mark β-catenin for proteosomal degradation causing a rise in its cytoplasmic concentration (Polakis 2012; Voloshanenko et al. 2013). However, predominantly not mutations, but rather epigenetic changes are the major pathogenic mechanisms in the Wnt-mediated carcinogenesis (Urakami et al. 2006). The Wnt pathway is essential for the maintenance of hematopoietic stem cells (HSC), and its overactivity can be observed in leukemia (Lento et al. 2013). More than half of the patients with breast cancer have an abnormal functioning of the Wnt signal pathway associated with reduced survival (Lin et al. 2000). Ligands and receptors of the canonical pathway are often overexpressed due to various mechanisms, while mutations much less frequently occur in pathway components (e.g., in β-catenin) (Yang et al. 2011). In ovarian cancer, for example, an overexpression of the non-canonical PCP pathway can be observed, which affects cell proliferation, cell cycle, and stemness (Asad et al. 2014).

There is no sharp dichotomy between the two main Wnt pathways as downstream signaling elements of certain ligands may overlap. For example, the (canonical) Wnt3a ligand- and the (non-canonical) Wnt5a ligand-initiated pathways can both agonize and antagonize with each other (Logan and Nusse 2004). Wnt5a may inhibit the canonical route by shifting the degradation of β-catenin to the Siah–APC pathway, thereby circumventing the conventional GSK-3β degradation pathway. Therefore, Wnt5a may act as an oncosuppressor (Topol et al. 2003). Wnt3a overexpression causes a translocation of the forkhead box protein M1 (FoxM1) into the nucleus where it forms a complex with β-catenin/TCF and induces the transcription of many downstream target genes (Abla et al. 2012). Interestingly, Wnt7a serves as a functional ligand for both the canonical and the non-canonical PCP pathways during embryogenesis, cell fate decision, and oncogenesis, thereby earning probably the most research interest among all Wnt ligands (Carmon and Loose 2008; Le Grand et al. 2009; Bikkavilli et al. 2015). Certain models suggest that the recruited co-receptors determine the specific activation of the canonical or non-canonical pathway (e.g., the non-canonical Wnt5a is also able to activate the canonical signaling pathway exclusively through LRP5/6 overexpression) (Suwala et al. 2016). How cells may discriminate among multiple Wnt ligands and what mechanism underlies selective recognition or decoding of a certain ligand was most elegantly presented for Wnt 7 in a recent study (Eubelen et al. 2018).

Activation and inhibition of Wnt pathways can be accomplished not only by their own ligands, but also by other regulatory molecules. Wnt inhibitor factor 1 (WIF1), frizzled-related protein family (FRPs), and Cerberus are secreted Wnt inhibitor molecules that prevent the binding of a soluble Wnt protein to its specific receptor, while Dickkopf (DKK) binds to one of the subunits of the Wnt receptor (Miller et al. 1999). WIF1 and FRPs block both the β-catenin and the Wnt/Ca^{2+} routes, but do not inhibit the PCP pathway. In contrast, DKK only regulates the β-catenin-dependent signaling, while Cerberus inhibits all three signaling pathways (Kawano and Kypta 2003). WIF1 expression has a negative influence on the invasiveness and migration capacity of tumor cells both in vitro and in vivo (Vassallo et al. 2016), and thus *WIF1* is considered as a strong tumor suppressor gene. Silencing of *WIF1* with the loss of its negative regulatory effect is often observed due to gene promoter deletion or hypermethylation in GBM (Lambiv et al. 2011).

Wnt Pathways in GBM and Its Molecular Subtypes (Table 1)

Several molecular pathways, including the Wnt pathway, define the biology, evolution, survival, and progression of gliomas. Positive modulation of a signaling pathway is often caused by switching off (e.g., by epigenetic silencing, gene deletions, or mutations) negative regulators (Götze et al. 2010; Lambiv et al. 2011). Aberrant signaling in both

Table 1 Effects of Wnt signaling in GBM

Expression patterns, mechanistic involvements, and broader interactions of Wnt pathway elements in GBM	References
Main biological effects of the Wnt pathways in GBM	
Define the biological behavior, evolution, survival, and progression of gliomas	Götze et al. (2010)
	Lambiv et al. (2011)
Contribute to the growth, aggressiveness, and invasive potential of GBM	Morris et al. (2013)
	Kamino et al. (2011)
Generate CSCs from differentiated cells	Kim et al. (2017)
Differential activity of Wnts in and around GBM	
Expression levels of Wnt genes are higher in the invasive zone than in the tumor center or in the normal brain tissue	Kahlert et al. (2012)
	Binda et al. (2016)
Molecular modulators of the Wnt pathways and GSCs	
FoxM1 interaction with β-catenin facilitates its nuclear translocation and promotes GSC self-renewal	Zhang et al. (2011)
	Suwala et al. (2016)
PLAGL-2 overexpression upregulates the canonical Wnt pathway elements β-catenin, Wnt6, FZD9, and FZD2	Gong and Huang (2012)
PLAGL-2 has a direct effect in regulating the GSC pool	Zheng et al. (2010)
SFRP1 is downregulated, while FZD2, 3, and 7 are upregulated in GSC	Sandberg et al. (2013)
Altered expressions of Wnt ligands and receptors	
Wnt3a and Wnt5a are overexpressed in GBM	Reis et al. (2012)
	Kaur et al. (2013)
Wnt5a is overexpressed in malignant gliomas, promotes GSC formation and GSC differentiation into endothelial-like cells, recruits existing endothelial cells, thereby facilitates GBM growth and invasion	Liu et al. (2011)
	Hu et al. (2016)
	Binda et al. (2016)
Wnt5a overexpression is higher in the mesenchymal than in the classical GBM subtype	Verhaak et al. (2010)
Most Wnt molecules have increased mRNA levels in GBM, while knock down of these molecules prevents tumor formation	Zhang et al. (2018) Paw et al. (2015)
Wnt receptors FZD2, FZD6, and FZD7 are elevated in GBM	Kamino et al. (2011)
EMT / PMT	
Wnt pathways contribute to EMT processes	Fu et al. (2011)
	Anson et al. (2012)
	Valenta et al. (2012)
EMT is a mechanism underlying dedifferentiation of differentiated cells to CSC	Yang and Weinberg (2008)
Hypoxic microenvironment promotes EMT and PMT	Piao et al. (2013)
	Segerman et al. (2016)
β-catenin by the upregulation of several target genes (e.g., ZEB1, Snail, Slug, and Twist) and transcription factors (e.g., LEF1) regulates EMT, and thus enhances tumor growth and invasion	Kahlert et al. (2012)
	Zhang et al. (2015)
Microenvironment in GBM	
Wnt3a and β-catenin induce tumor-associated microglial cells by the upregulation of several molecules (ARG-1 and STI1, IL-10) to evade immune response	Matias et al. (2018)
Wnt5a induces the glioma-associated microglia to attain proinflammatory characteristics, recruits existing endothelial cells, and promotes tumor angiogenesis	Dijksterhuis et al. (2015) Hu et al. (2016)
Glioma-associated reactive astrocytes show EMT and active Wnt/β-catenin signaling, increased migration and invasion, upregulation of MMPs and altered expression of adhesion molecules serving tumor spread. Tumor invasion and growth may be manipulated by blocking these molecules	Lu et al. (2016)
Wnt7 in Olig2+ oligodendrocyte precursor-like glioma regulates single-cell vessel co-option and contributes to blood–brain barrier integrity and temozolomide response	Griveau et al. (2018)
Interaction between Wnt pathways molecules and the IDH R132H GBM subgroup marker	
IDH1 R132H mutation is in an inverse correlation with the expression level and nuclear localization of β-catenin, and is associated with decreased proliferation, increased apoptosis, and decreased invasive potential of GBM cells	Cui et al. (2016)
IDH1 R132H upregulates negative regulators of Wnt/β-catenin pathway, while downregulates several effector and target molecules	Cui et al. (2016) Yao et al. (2018)
IDH mutant tumors have lower numbers of GSCs than IDH wild-type tumors	Yao et al. (2018)

Table 1 (continued)

Expression patterns, mechanistic involvements, and broader interactions of Wnt pathway elements in GBM	References
IDH1 R132H downregulates the Wnt transcription factor TCF4 and through that Ki-67, leading to lower invasive and migratory potential of mutant gliomas	Denysenko et al. (2016)
	Wang et al. (2016)

the canonical and non-canonical pathways contributes to the growth and aggressiveness of GBM. Defective activation of the canonical β-catenin-mediated pathway is closely related to the formation and maintenance of GSCs (Morris et al. 2013), while aberrant regulation of the non-canonical Wnt pathway increases the invasive potential of the tumor (Kamino et al. 2011). Wnt/β-catenin activation is also needed for generating CSCs from differentiated cells (Kim et al. 2017).

Immunohistochemistry analyses showed that the canonical as well as the non-canonical Wnt pathway molecules are most highly expressed in the infiltration zone as compared to the center of tumors or to the normal appearing brain tissues. These observations suggest that the Wnt pathways define the invasion potential of cancerous cells, and drive tumor growth, recurrence, and progression (Kahlert et al. 2012; Binda et al. 2016).

The activity level of Wnt/β-catenin pathway is not only influenced by the ligand-receptor engagement. For example, FoxM1 interacts with β-catenin and signal transducer and activator of transcription 3 (STAT3) to promote GBM-GSC renewal (Suwala et al. 2016). Furthermore, FoxM1 and β-catenin form a functional complex with TCF4 and promote GBM-initiating cell (GIC) self-renewal and tumorigenesis (Zhang et al. 2011). In addition, numerous other proteins have increased levels influencing the Wnt pathways and defining biological characteristics of GBM. Such examples include the pleomorphic adenoma gene-like 2 (PLAGL-2) protein and the frizzled-4 (FZD-4) receptor (both involved in the development of GSCs) that enhance resistance to irradiation, and the expression of VEGF promoting angiogenesis and vasculogenesis (Zheng et al. 2010; Jin et al. 2011; Liu et al. 2015). Amplification of *PLAGL-2* frequently occurs in primary GBM, and overexpression of *PLAGL-2* induces an upregulation of the canonical Wnt signaling elements β-catenin, Wnt6, FZD9, and FZD2 (Gong and Huang 2012). Inhibition of the PLAGL-2 decreases cell stemness. Independent of the Wnt pathway, PLAGL-2 also has a direct effect in maintaining and regulating the GSC pool (Zheng et al. 2010). Another example of molecular dysregulation of the Wnt pathway is presented by Sandberg et al. (2013) who identified significant downregulation of the secreted frizzled-related protein (SFRP1) and upregulation of the FZD receptors (FZD2, 3, and 7) in GSC.

Altered expressions of Wnt pathway ligands and receptors have been noted in numerous studies in GBM. Based on the

TCGA data, Wnt3a (an initiator of the canonical pathway) and Wnt5a (an initiator of the non-canonical pathway) are overexpressed in GBM, and also in grade II and III gliomas (Reis et al. 2012; Kaur et al. 2013). Similarly, the expression of FZD2, FZD6, and FZD7 receptors are elevated in these tumors (Kamino et al. 2011). Overall, the mRNA expression levels of most Wnt genes are markedly increased in GBM as compared to normal brain tissues (Zhang et al. 2018), and the expression levels of Wnt1, β-catenin, and cyclin D1 correlate with the glioma grade (Utsuki et al. 2002; Liu et al. 2011). In contrast, knockdown of Wnt1 resulted in smaller and non-invasive intracranial tumors, while knockdown of Wnt3a completely prevented tumor formation (Paw et al. 2015). Quantitative real-time PCR analyses also revealed an overexpression of Wnt5a in the most infiltrative malignant gliomas consistent with the TCGA data (Binda et al. 2016). Integrated transcriptomic and epigenomic analyses pointed to a transcriptional program driving Wnt5a-mediated GSC differentiation into endothelial-like cells (GdECs). Peritumoral satellite lesions serve as a niche and support the invasive glioma cell migration away from the core tumor region and facilitate tumor growth via Wnt5-mediated recruitment of existing endothelial cells, and thereby enhance GSC's self-renewal (Hu et al. 2016). In addition, the Wnt5a ligand alone can modify the tumor-promoting stem-like characteristic (TPC) pattern, cause a global phenotypic shift of TPCs, and increase the invasiveness of these cells in GBM. The Wnt5a-driven new molecular TPCs phenotype is associated with poor prognosis and shorter survival time (Liu et al. 2011; Binda et al. 2016). Likely related to these biological features, Wnt5a overexpression is more pronounced in the mesenchymal than in the classical subtype of GBM as reflected by TCGA analyses (Verhaak et al. 2010).

The so-called epithelial–mesenchymal transition (EMT) is an important early event in metastasis formation by many cancers, and in the invasive growth of gliomas, in which the Wnt/β-catenin signaling pathway has established roles (Fu et al. 2011; Valenta et al. 2012). During EMT, the epithelial tissue undergoes specific genetic and biochemical changes with the resultant generation of a mesenchymal tissue (Yang and Weinberg 2008). Antiangiogenic therapies (e.g., anti-VEGF bevacizumab) reduce angiogenesis and vascularization, and unfortunately also create a hypoxic microenvironment that promotes EMT (Piao et al. 2013). Piao et al. (2013) showed that hypoxia contributes to an inflammatory environment and indirectly promotes mesenchymal

transition. Chronic use of an antiangiogenic therapy induces treatment resistance while glioma cells undergo proneural to mesenchymal shift (PMT), the EMT equivalent in GBM (Piao et al. 2013). EMT and PMT may be induced by additional extrinsic factors such as tumor necrosis factor α (TNF-α) and the Wnt/Ca2 + pathway-induced NF-κB (Segerman et al. 2016). EMT is also one of the key mechanisms of dedifferentiation of differentiated cells to CSCs (Yang and Weinberg 2008). For the initiation of EMT, several specialized molecules and high levels of β-catenin are needed (Fu et al. 2011; Anson et al. 2012; Valenta et al. 2012). During activation of the canonical Wnt pathway in EMT, β-catenin accumulates in the nucleus where it regulates several target genes (e.g., *ZEB1, Snail, Slug,* and *Twist*) and causes upregulation of transcription factors (e.g., LEF1) involved in tumor invasion and progression (Kahlert et al. 2012; Zhang et al. 2015). Cells involved in EMT will develop increased migratory capacity and acquire resistance to apoptosis and irradiation (Kalluri and Weinberg 2009). Different Wnt ligands have different potentials in shaping and maintaining cancers (MacDonald et al. 2009; Anastas and Moon 2013).

High-grade gliomas rarely metastasize outside of the central nervous system, but do migrate within the brain along blood vessels, neuronal processes, or glial cells, facilitated by a complex interaction with their microenvironment. Cells transformed by EMT acquire a capability of breaking down integrity of the extracellular matrix (ECM) by an overproduction of MMPs and migrate in the intercellular space with the help of overexpressed adhesion molecules (Kim et al. 2017). Normal stem cells can be found in a so-called "stem cell niche" that regulates stemness, proliferation, and apoptosis, and have a complex interaction with their microenvironment involving stromal cells (mesenchymal and immune cells), the vascular network, extracellular matrix, and soluble factors (Borovski et al. 2011). GSCs form the "GSC niche" that also exerts a complex interaction with its microenvironment for developing unique tumor-promoting capabilities and protective roles against various therapies. Recent observations reveal that the GBM-derived Wnt3a induces M2-like microglial cells through β-catenin signaling, and upregulation of several molecules (ARG-1 and STI1 followed by upregulation of interleukin-10 and downregulation of interleukin 1β) acting in concert to enhance migration capability of GBM and evasion of the immune response (Matias et al. 2018). Wnt5a is also associated with an increased presence of glioma-associated microglia and monocytes, but with enhanced expression of the major histocompatibility complex Class II (MHC II) molecules, suggesting a proinflammatory profile in the microenvironment of these tumors (Dijksterhuis et al. 2015). In addition, astrocytes are activated in the microenvironment of high-grade gliomas. These reactive astrocytes are characterized by EMT transition, enhanced migration and invasion, and

the decrease of E-cadherin and increase of vimentin and MMP expression serving tumor invasion (Lu et al. 2016). The canonical Wnt/β-catenin signaling pathway was found to be activated in these tumor-associated astrocytes, which could be manipulated by Wnt/β-catenin pathway inhibitors. Wnt7 also deserves here a brief note as it was recently shown to regulate Olig2 + oligodendrocyte precursor-like glioma single-cell vessel co-option, blood–brain barrier integrity, and the temozolomide treatment response (Griveau et al. 2018). These observations indicate, at least in part, that the glioma vascular and microenvironment interactions are very complex involving a multitude of Wnt pathway elements interacting with other molecular drivers of EMT/PMT in GBM.

Explorations of the interactions between Wnt pathway elements and the IDH1 R132H GBM subgroup marker only recently emerged, but have already revealed important observations that further elucidate glioma pathogenesis. As discussed above, the determination of IDH status (where the mutant status correlates with the proneural subtype) has become part of clinical histopathological evaluations for GBM (as for gliomas of all grades), but the determination of other GBM subgroup markers remains explorative at the present time (Louis et al. 2016; Nagy et al. 2017). IDH1 R132H mutant gliomas have significantly lower proliferation, migration, and invasion capabilities, and have higher number of apoptotic cells, as compared to their IDH wild-type counterparts (Xiang et al. 2017). Reactive oxygen species (ROS) levels in cancer cells are higher than in normal cells due to oncogenic stimulation. ROS promotes the proliferation potential and survival of cancerous cells, but above a certain threshold, ROS can reduce or inhibit tumor growth and proliferation by promoting cell death (Xiang et al. 2017). Basal level of ROS combined with the ROS due to an IDH-1 mutation raise chemosensitivity and apoptotic potential of cancer cells (Houillier et al. 2010; Shi et al. 2015). Cui et al. (2016) studied two glioblastoma cell lines (U87; U251) which were transfected with an empty lentiviral vector, or with a vector containing the R132H mutant or the wild-type IDH. The authors showed that the presence of IDH1 R132H mutation is in an inverse correlation with the expression level and nuclear localization of the Wnt mediator β-catenin in gliomas (Cui et al. 2016). Both in vitro and in vivo (mouse xenograft model) analyses confirmed that overexpression of the R132H IDH mutant decreased the proliferation rate, elevated the apoptotic activity, and significantly decreased the migration and invasive potential of these modified GBM cell lines (Cui et al. 2016). At molecular level, the R132H IDH1 mutation significantly reduced the canonical Wnt/β-catenin signaling as GBM cells with the mutation upregulated negative regulators of Wnt/β-catenin pathway (e.g., DKK1 and APC), while downregulated several effector and target molecules (e.g., β-catenin and TCF4/LEF1

transcriptional factors) (Cui et al. 2016). Yao et al. (2018) examined the correlation between the GSC invasive potential and IDH status. These studies revealed lower numbers of CD133^{+} positive GSCs and overall GSCs in IDH mutant than in IDH wild-type tumors. The IDH mutant group also had significantly decreased expression levels of mRNA and protein for β-catenin as well as decreased levels of TCF4 and LEF1 transcriptional factors, when compared to those of IDH wild-type tumors (Yao et al. 2018). These observations, showing an inverse correlation between the IDH status and canonical Wnt pathway, arose in consensus from immunofluorescence, Western blot, and microarray analyses (Cui et al. 2016; Yao et al. 2018). TCF4 has a strong effect on the expression level of Ki-67 (Denysenko et al. 2016) and the IDH R132H mutation reduces the KI-67 index in all glioma subtypes (Wang et al. 2016). Therefore, the invasive and migratory potential of tumor cells are likely hindered in IDH1 R132H positive gliomas due to this mutation that decreases the expression level of TCF4, and through that downregulates the Ki-67 protein. These findings altogether suggest that the better prognosis of glioma patients with the IDH1 R132H mutation may be related, at least in part, to the downregulation of the Wnt/β-catenin pathway.

Therapeutic Strategies Targeting Wnt Signaling and Interacting Molecules

Recurrence of GBM is strongly influenced by the presence of residual GSCs after therapy. Therefore, targeting these cells post-surgery likely would enhance therapeutic efficacy (Safa et al. 2015). Development of an effective multi-target strategy for GBM is challenging and success has mostly been achieved only in preclinical models. As potential interventions, inhibition of growth factor receptors, regulatory and signaling molecules (EGFR, CD95 and mTOR proteins), or drivers of angiogenesis and modifiers of stroma (e.g., VEGF, TGFβ, and c-Met) have been considered in order to prevent tumor growth and invasion or to enhance immune response (Debus and Abdollahi 2014). By inhibiting mTOR and using all-trans retinoic acid (ATRA), GSCs could be forced to differentiate and to become more sensitive to therapy (Friedman et al. 2013). Since microRNAs play an important regulatory role in tumors, a modification of the microRNA network could modulate apoptotic routes, differentiation, proliferation, migration, and drug resistance of GSCs (Gao and Jin 2014; Kouri et al. 2015). MiR-182, for example, blocks Bcl2-like12 (Bcl2L12), c-Met, and hypoxia-inducible factor 2α (HIF2α) protein, which result in a decreased GSC spheroid size and stemness in vitro. In animal models, the intravenously injected 182-SNAs (synthesized miR-182-based spherical nucleic acids) with long construct lifetime and without significant immunogenicity,

pass through the blood–brain barrier of orthotopic GBM xenografts and reduce tumor growth while increase survival time (Kouri et al. 2015). MiR-101 also functions as a tumor suppressor in GBM (Visani et al. 2014; Yao et al. 2015) as it reduces the invasive activity, proliferation, and migration potential of GSC by inhibiting Kruppel-like factor 6 (KLF6) transcriptional factor (Yao et al. 2015). Finally, miR-218 can suppress the mRNA of the LEF1 transcriptional factor that is upregulated by β-catenin. Inhibition of LEF1 leads to a decreased activity of MMP-2, MMP-7, and MMP-9 in vitro, which thereby causes diminished invasion and metastatic potential of cancer (Lo et al. 2009).

As discussed above, the Wnt pathway increases resistance to chemotherapy, therefore, its molecular components may be plausible direct supplementary treatment targets. Beside the natural inhibitor proteins (WIF, DKK, FRP, etc.), the candidate drugs that inhibit the Wnt signaling pathway can be divided into three main groups: non-steroidal anti-inflammatory drugs (NSAIDs), small molecule chemical inhibitors, and monoclonal antibodies (Lee et al. 2016). Aspirin, sulindac, and the selective COX2 inhibitor celecoxib are NSAIDs that could help to reduce the Wnt activity in tumors. Aspirin, a traditional NSAID, has a chemoprotective effect mainly in colon cancer (Kahn 2014). However, in experimental systems, aspirin also reduces the proliferation rate and invasiveness of GBM tumor cells and increases the number of apoptotic cells, mainly by the diminished activity of TCF/LEF transcription factor and Wnt target genes (*c-MYC, Cyclin D1*) (Lan et al. 2011). Small molecular inhibitors may be even a more potent and effective way to reduce the activity of the Wnt pathways. Some of these molecules are already undergoing clinical testing. LGK974 blocks the activity of porcupine protein essential for the palmitoylation of Wnt ligand proteins, with a resultant reduction of their secretion or limited binding to their receptors (Suwala et al. 2016). In in vitro preclinical trials, LGK974 reduced the canonical Wnt pathway activity, tumor growth, and proliferative capacity of stem-like cancer cells in three glioblastoma cell lines (Kahn 2014; Kahlert et al. 2015). SEN461 is a promising small chemical inhibitor molecule that prevents the proteosomal degradation of the AXIN scaffold protein. Stable AXIN level increases the levels of phosphorylated and ubiquitinated cytoplasmic β-catenin (Lee et al. 2016; Suwala et al. 2016). PIR-724 is a potent inhibitor of the canonical Wnt pathway by blocking the interaction between β-catenin and its co-activator CBP (CREB-binding protein) thereby reducing the expression levels of β-catenin target genes (Kim et al. 2014). The goal of Wnt pathway-directed immune therapy is to neutralize Wnt ligands and the FZD receptors by monoclonal antibodies. A Wnt-1 - specific monoclonal antibody successfully suppressed cellular growth of lung cancer both in *in vitro* and in vivo conditions. Monoclonal antibody specific to

secreted frizzled-related protein 2 (SFRP2) reduced cancer cells migration in angiosarcoma (He et al. 2004; Fontenot et al. 2013; Lee et al. 2016). Also currently in clinical trials is OMP-18R5, another monoclonal antibody that binds to several FZD receptors in solid tumors (e.g., breast cancer) (Kim et al. 2014). Ipafricept (OMP-54F28) is a recombinant fusion protein with an extracellular part of FZD8 fused to a human IgG1 Fc fragment that binds all Wnt ligands in solid tumors (Kim et al. 2014; Jimeno et al. 2017). An effective therapy for GBM with mutant *IDH1* or *IDH2* likely will require selective inhibition of the mutant enzyme and the production of D2HG oncometabolite (Wu et al. 2018). In addition, the G-CIMP epigenetic phenotype of *IDH* mutant gliomas may be reversed by demethylation of abnormally hypermethylated tumor suppressor regions in order to prevent tumor growth, invasion, and recurrence (Turcan et al. 2013). One may expect that combine targeting of the IDH mutant enzyme and elements of Wnt signaling may result in a more powerful strategy that merit further studies in IDH1 R132H positive gliomas. While altogether the diversity and speed of therapeutic developments are very impressive in GBM, even this brief overview of selected therapeutic agents relevant to GSCs and their most critical signaling pathways reveals that almost all experimental drugs are presently in preclinical stages with only a few reaching human testing.

Conclusion

Here we surveyed data focusing on functional interconnections among a few essential molecules and important pathways regulating GBM development and behavior. We have emphasized the roles of the Wnt pathway in the context of CSC/GSC, tumor biology and molecular subtypes of GBM. Recent advancements in the field not only provide more insight into molecular gliomagenesis, but also reveal new opportunities for targeted and combined therapies. A better understanding of cell specificity, selective recognition, and signaling of Wnt molecules along with their receptors and co-receptors in normal stem cells and CSCs or GSCs will likely facilitate a more effective manipulation of tumor development. Further explorations of biological consequences of Wnt pathway activation in various cell types and conditions may be another area of research to serve translation. One may also keep in mind that Wnt ligand and receptor interactions not only frequently exert pleiotropic effects (with multiple receptor and cell targets), but their signaling is often redundant (the same end result caused by more than one ligand and pathway), making in vivo interventions challenging and prompting us to promote combined target strategies. Unfortunately, there are still too many open questions to overcome when starting from intelligent drug design and continuing with the testing of their desired

effects in cell culture, animal models, and humans. Much evidence suggests that focusing not only on the tumor but also on its microenvironment will be essential to overcome GBM. While there are many difficulties that remain to be solved, it is already apparent that a successful elimination of GBM will require complex and inventive strategies, and the core pathways highlighted here likely will be part of these approaches.

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