

# Molecular Neurooncology and Neoangiogenesis of Malignant Gliomas

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**Abstract** Malignant gliomas are the most common and most aggressive primary brain tumors in adults. Advances in surgery, radiotherapy and chemotherapy only have a minor impact on the natural course of these tumors. Due to the dismal prognosis of malignant glioma patients, there is an urgent need for new innovative treatments based on a better understanding of the molecular mechanisms of gliomagenesis. Many growth factors, growth factor receptors – usually receptor tyrosine kinases – and receptor-associated intracellular signaling pathways are critically involved in glioma growth, invasiveness and tumor neovascularization.

Therefore, this chapter highlights the most important signaling pathways involved in initiation and progression of malignant gliomas. The knowledge of these pathways is the rationale of several concepts of new innovative molecular therapies in modern neurooncology.

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## Abbreviations

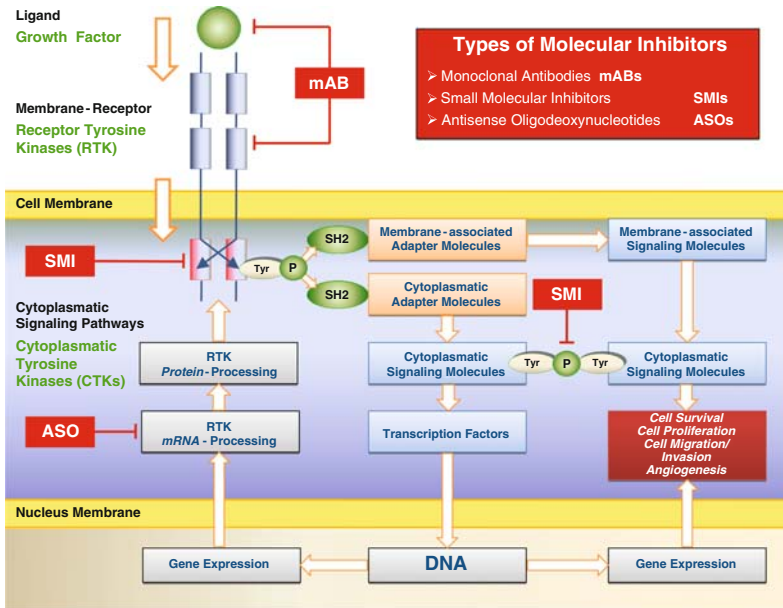
AAC	Anaplastic astrocytoma
AG	Anaplastic glioma
AOD	Anaplastic oligodendroglioma
ASO	Antisense oligonucleotide
CIR	Clinical response
CR	Complete response
DAG	Diacylglycerol
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal regulated kinase
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GBM	Glioblastoma multiforme
GF	Growth factor
Grb2	Growth factor receptor-bound protein 2
IGF	Insulin growth factor
IGFR	Insulin growth factor receptor
LT	Ligand linked targeted toxins
mAB	Neutralizing monoclonal antibody
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
mTOR	Mammalian target of rapamycin
PD	Progressive disease
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidyl-inositol-3-kinase
PKC	Proteinkinase C
PLC	Phospholipase C
PR	Partial response
rAAC	Recurrent anaplastic astrocytoma
Ras-GAP	GTPase-activating protein of Ras
Ras-GTP	Ras-guanosine-triphosphate, active Ras
rGBM	Recurrent glioblastoma multiforme
rHGG	Recurrent high grade Glioma
RTK	Receptor tyrosine kinase
SD	Stable disease
SR	Soluble receptor
SMI-R	Small molecule inhibitor of receptors
SMI-S	Small molecule inhibitor of intracellular signaling molecules
TGF	Transforming growth factor
TGFR	Transforming growth factor receptor

## 1 Molecular Pathways in Malignant Gliomas

Malignant gliomas are the most common primary brain tumors of the central nervous system, resulting from the transformation of a glial or supportive (stem) cell. The term “malignant glioma” summarizes different histological subtypes of anaplastic gliomas WHO grade III (anaplastic astrocytoma, AAC; anaplastic oligodendroglioma, AOD; anaplastic oligoastrocytoma, AOA) and glioblastoma multiforme (GBM) WHO grade IV.

Despite improvements in neurosurgical techniques, radiation and chemotherapy during the past three decades, little progress has been made in the treatment of malignant gliomas, and therapy remains mostly palliative. Since the study by Walker et al. (1980), which showed an increased survival of patients receiving radiotherapy after surgery compared to surgery alone, adjuvant radiotherapy became a first-line standard treatment for GBM patients in many countries. As a next step, combined radio- and chemotherapy after surgical intervention has become a standard treatment for malignant glioma patients in the 1990s. Furthermore, a metaanalysis of 3,004 malignant glioma patients from 12 randomized controlled trials showed a modest but statistically significant prolongation of the 1-year survival rate (from 40 to 46%) for adjuvant nitrosourea-based chemotherapy added to radiotherapy after surgical resection (Stewart 2002). In 2005, Stupp et al. published the results of a large clinical trial examining the role of adjuvant *Temozolomide* chemotherapy in the management of newly diagnosed GBM. Currently, this study represents the first-line standard therapy regime for GBM, which includes maximal surgical tumor resection followed by *Temozolomide* applied concomitant and adjuvant to local field radiotherapy. This treatment results in a mean overall survival time of only 9–15 months from the time of diagnosis (Stupp et al. 2005). Therefore, there is an urgent need to explore new treatment options for brain tumors.

In the past three decades, the discovery of oncogenes and tumor suppressor genes defined biological hallmarks of cancer (Hanahan and Weinberg 2000; Vogelstein and Kinzler 2004). Advances in molecular biology of tumors have enhanced the understanding of carcinogenesis, which results from a deregulation in the network of extracellular, membrane-associated and intracellular signaling cascades. Oncogene amplification or rearrangement, as well as aberrant regulation of structurally intact genes or gene-mutations leading to an unregulated function, result in overexpression, autocrine/paracrine cell stimulation and autonomic protein function (Maher et al. 2001). Growth factors (GFs), growth factor receptors (GFRs) – usually receptor tyrosine kinases (RTKs) – and GFRs-mediated signal transduction pathways have been identified to play an essential role in tumor initiation, tumor growth and tumor angiogenesis (Ohgaki et al. 2004; Ohgaki and Kleihues 2005; Shawver et al. 2002; von Deimling et al. 1995; Zwick et al. 2002), affecting fundamental cellular processes, such as cell differentiation, proliferation, survival, migration and metabolism (Aaronson 1991; Cantley et al. 1991). Brain tumor cells are known to have the capacity to secrete GFs and overexpress the



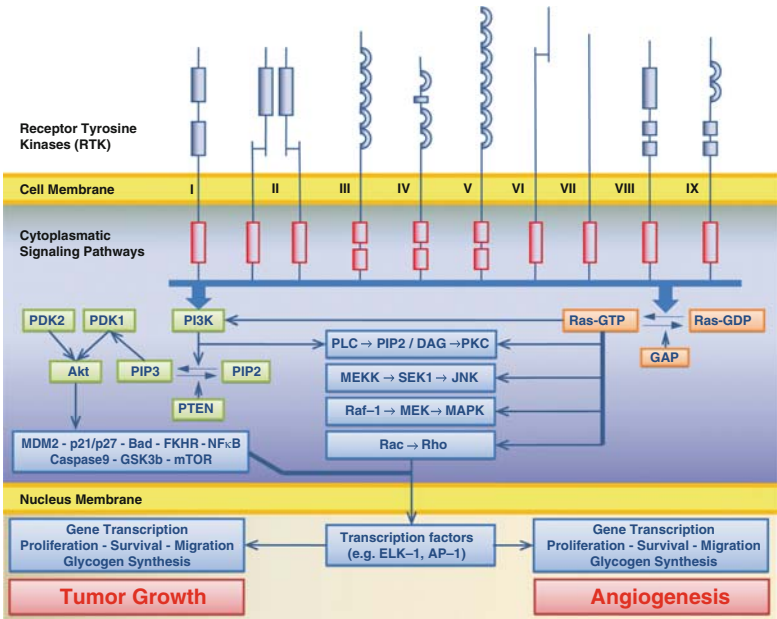
**Fig. 1** Principles of growth factor receptor signaling pathways and targeted therapy approaches

corresponding growth factor receptors, allowing for both autocrine and paracrine stimulatory loops. This excessive stimulation affects the secreting tumor cell and surrounding tumor cells, as well as regional glial, vascular smooth muscle and vascular endothelial cells (Guha et al. 1995; Maher et al. 2001).

These new molecular biological insights stimulated the development of a new generation of cancer therapeutics of more targeted and specific treatment modalities by blocking growth and spreading of cancer by interfering with specific molecules that play a key role in cancer development and progression (Hanahan and Weinberg 2000) (Figs. 1 and 2). Such treatment approaches include immunotherapy using monoclonal antibodies (mABs), ligand linked targeted toxins, small molecule inhibitors of receptors (SMI-R) and intracellular signaling molecules (SMI-S) as well as antisense oligonucleotides (ASO) (Strebhardt and Ullrich 2008), which result in personalized and tailored therapy approaches precisely targeting the specific molecular defects of a tumor.

### 1.1 Genetic Pathways of Primary (De Novo) and Secondary Glioblastomas

In malignant gliomas, molecular pathways and gene transcription regulations are expressed very heterogeneously. They differ significantly in RNA and protein expression profiles and in their pattern of promoter methylation (Tables 1 and 2)



**Fig. 2** Growth factor receptors and their associated intracellular signaling pathways

**Table 1** Incidence, age, gender and survival in primary and secondary glioblastomas. Modified from Ohgaki and Kleihues (2007)

	Primary GBM	Secondary GBM	References
Incidence rate <sup>a</sup>	3.531	0.199	
Incidence rate <sup>b</sup>	2.575	0.167	
Mean age	62 years	45 years	Ohgaki and Kleihues (2005)
Male/female ratio	1.33	0.65	Ohgaki and Kleihues (2005)
Clinical history	< 3 months 68%	From grade II 5.3 years	Ohgaki and Kleihues (2005)
	3–6 months 16%		
	> 6 months 16%	From grade III 1.4 years	
	mean 6.3 months		
Survival	Grad IV Median 4.7 months	Grad IV Median 7.8 months	Ohgaki et al. (2004)

<sup>a</sup>Adjusted to the European standard population (per 100,000 persons per year)

<sup>b</sup>Adjusted to the World standard population (per 100,000 persons per year)

(Furuta et al. 2004; Godard et al. 2003; Tso et al. 2006). This has significant implications, since different molecular signatures may not only affect sensitivity to radio- and chemotherapy, but also to targeted therapies.

*Primary glioblastomas* represent the majority of GBMs (95%) and develop rapidly de novo without clinical or histological evidence of a less malignant precursor lesion (Ohgaki et al. 2004) by resulting from the acquisition of multiple genetic alterations. Primary GBMs mainly affect the elderly and are genetically

**Table 2** Genetic and epigenetic changes and expression profiles in primary and secondary glioblastomas. Modified from Ohgaki and Kleihues (2007)

Genetic alterations	Primary GBM	Secondary GBM	Low grade glioma	References
<i>TP53</i> mutations	28%	65%		Ohgaki et al. (2004)
<i>EGFR</i> amplification	36%	8%		Ohgaki et al. (2004)
<i>PTEN</i> mutations	25%	4%		Ohgaki et al. (2004)
<i>p16<sup>INK4a</sup></i> deletion	31%	19%		Ohgaki et al. (2004)
LOH 1p	12%	15%		Nakamura et al. (2000)
LOH 10p	47%	8%		Fujisawa et al. (2000)
LOH 10q	47%	54%		Fujisawa et al. (2000)
	70%	63%		Ohgaki et al. (2004)
LOH 13q	12%	38%		Nakamura et al. (2000)
LOH 19q	6%	54%		Nakamura et al. (2000)
LOH 22q	41%	82%		Nakamura et al. (2005)
<i>Promoter methylation</i>				
<i>p14<sup>ARF</sup></i>	6%	31%		Nakamura et al. (2001)
<i>p16<sup>INK4a</sup></i>	3%	19%		Nakamura et al. (2001)
MGMT	36%	75%		Komine et al. (2003)
TIMP-3	28%	71%		Nakamura et al. (2005)
<i>Expression profiles</i>				
EGFR (HER1, ErbB1) <sup>a</sup>	63%	10%		Watanabe et al. (1996)
EGFR (HER1, ErbB1) <sup>b</sup>	High	Low		Furuta et al. (2004)
EGFR amplification	40%	Low	Low	Ohgaki et al. (2004)
EGFR overexpression	>60%	<10%		Watanabe et al. (1996)
EGFRvIII	47% of EGFR-pos GBM			Ekstrand et al. (1994); Frederick et al. (2000)
HER2/neu (ErbB2)	High	Low	Low	Mineo et al. (2007)
PDGFR- $\alpha$	Overexpressed ++	Overexpressed +++	Overexpressed +	Di Rocco et al. (1998); Hermanson et al. (1992)
PDGF-A	High +	High +++	Low	Hermanson et al. (1992)
PDGF-B	High +	High +++	High +	Hermanson et al. (1992)

(continued)

Table 2 (continued)

Genetic alterations	Primary GBM	Secondary GBM	Low grade glioma	References
PDGF-AB <sup>c</sup>	High +	High +++	Low	Karcher et al. (2006)
TGF-βRI/II	High		Low	Jennings and Pietenpol (1998); Platten et al. (2001)
TGF-α	High		Low	Maier et al. (2001); Samuels et al. (1989)
TGF-β	High		Low	Jennings and Pietenpol (1998); Platten et al. (2001)
FGF (acid, basic)	High		Low	Dunn et al. (2000); Stefanik et al. (1991)
FGFR	High		Low	Dunn et al. (2000); Stefanik et al. (1991)
VEGF <sup>c</sup>	High	Low		Karcher et al. (2006)
VEGF fms-related TK 1 <sup>d</sup>	High	Low		Godard et al. (2003)
MMP-9 <sup>a</sup>	69%	14%		Choe et al. (2002)
Ras-GTP	High		Low	Feldkamp et al. (1999); Guha et al. (1997)
PTEN hemizygous deletion	60–80%			Vazquez and Sellers (2000)
PTEN mutations/LOF	15–50%	<10%	Rare or absent	Vazquez and Sellers (2000)
PTEN loss of expression	65%	<10%	Rare or absent	Knobbe et al. (2002); Sasaki et al. (2001)
TP53 <sup>a</sup>	37%	97%		Watanabe et al. (1996)

<sup>a</sup>Immunohistochemistry (IHC)

<sup>b</sup>2D Gel Electrophoresis

<sup>c</sup>Enzyme-linked immunosorbent assay (ELISA)

<sup>d</sup>cDNA microarray

<sup>e</sup>Reverse transcriptase-polymerase chain reaction (RT-PCR)

characterized by loss of heterozygosity 10q (*LOH 10q*, 70% of cases), *EGFR* amplification (36%), *p16<sup>INK4a</sup>* deletion (31%), and *PTEN* mutations (25%) (Tables 1 and 2). In contrast, *LOH 10p* or *complete loss of the entire chromosome 10* is typically present in primary GBMs (Fujisawa et al. 2000). Several LOH studies identified at least three commonly deleted loci, i.e., 10q23–24 (*PTEN*), suggesting the presence of several tumor-suppressor genes that may play significant roles in the pathogenesis of GBMs (Ichimura et al. 1998; Rasheed et al. 1995).

*Secondary glioblastomas* develop slowly through progression from low-grade diffuse astrocytoma (WHO grade II) or AAC (WHO grade III) and affect younger patients (Table 1). The diagnosis of secondary glioblastoma requires clinical (neuroimaging) or histological evidence of an evolution from a less malignant astrocytoma. In 60% of precursor low-grade astrocytomas, TP53 mutations are the most frequent and earliest detectable genetic alterations. During progression to GBM, additional mutations accumulate, including LOH 10q25, which is the most frequent genetic alteration occurring in both primary and secondary GBM at similar frequencies (60–80%) (Table 2) (Fujisawa et al. 2000; Ichimura et al. 1998; Ohgaki et al. 2004; Rasheed et al. 1995).

Secondary GBM show a higher frequency of promoter methylation than primary GBM (Table 2). *O<sup>6</sup>-Methylguanine-DNA methyltransferase* (MGMT) is a repair protein that specifically removes promutagenic alkyl groups from the O<sup>6</sup> position of guanine in DNA. MGMT therefore protects cells against carcinogenesis induced by alkylating agents (Goth and Rajewsky 1974; Margison and Kleihues 1975). In contrast, repair of O<sup>6</sup>-alkylguanine adducts by tumor cells has been implicated in drug resistance, because it reduces the cytotoxicity of alkylating chemotherapeutic agents, including *Temozolomide* (Belanich et al. 1996). Loss of MGMT expression caused by methylation of promoter CpG islands (Qian and Brent 1997; Watts et al. 1997) was detected in 75% of secondary GBM, significantly more frequently than in primary GBM (36%) (Watts et al. 1997). The difference in frequency of MGMT methylation between primary and secondary GBM is clinically relevant, because patients with GBM containing a methylated MGMT promoter were shown to have a substantially greater benefit from adjuvant *Temozolomide* treatment (Hegi et al. 2005).

## 1.2 Major Signaling Pathways Regulating Tumor Growth

### 1.2.1 EGF/EGFR Signaling Pathway

The polypeptide *epidermal growth factor* (EGF) is the monomeric ligand of the *epidermal growth factor receptor* (EGFR, HER1, ErbB1) (Carpenter and Cohen 1979; Maher et al. 2001). In addition, EGFR is also capable of binding other monomeric ligands, such as TGF- $\beta$ , amphiregulin, heparin-binding-EGF, betacellulin and epiregulin (Arteaga 2001; Hackel et al. 1999; Hubbard and Till 2000) (Figs. 1 and 2). The EGFR is a member of the HER-family (HER1–4). The

homo- or heterodimeric partner of the EGFR will determine the sites of autophosphorylation and the signaling molecules that associate with the receptor (Fig. 1). Therefore, the specificity and potency of the signaling output will vary depending on the identity of the coreceptor. High HER2 expression was demonstrated in primary (de novo) GBM. In contrast, secondary GBM, and similarly gliomas grade III, express HER2 with low intensity (Mineo et al. 2007).

The EGF/EGFR pathway is a key signaling in the transformation process of various solid tumors (Frederick et al. 2000; Hurtt et al. 1992; Maher et al. 2001; von Deimling et al. 1995), including the development of primary GBMs (Kita et al. 2007). In nontransformed cells, EGFR concentrations and signaling activity are tightly controlled. In malignant glioma cells, the membrane density of EGFR is often excessive and the corresponding signaling pathways (PI3K-Akt, PLC-DAG-PKC, Ras-Raf1-MEK-MAPK) are hyperactive (Arteaga 2001; Frederick et al. 2000). GBMs are known to overexpress EGF, TGF- $\alpha$  and EGFR, consistent with autocrine and paracrine stimulatory loops.

*EGFR gene amplification* occurs in ~40% of primary GBM (Ekstrand et al. 1992; Ohgaki et al. 2004), but rarely in secondary glioblastomas (Ohgaki et al. 2004) (Table 2). *EGFR overexpression* is more common in primary glioblastomas (>60%) than in secondary glioblastomas (<10%) (Watanabe et al. 1996). All primary glioblastomas with EGFR amplification show EGFR overexpression, and 70–90% of those with EGFR overexpression have EGFR amplification (Biernat et al. 2004; Tohma et al. 1998).

In GBM, amplification of the wild-type EGFR gene appears to be a precursor to subsequent *mutations of EGFR*, which usually involve intragene deletions (Frederick et al. 2000). The most frequent type is the variant 3 (*EGFRvIII*) with deletion of exons 2–7, thereby removing the ligand-binding domain of the receptor. This mutation occurs in 67% of EGFR-positive tumors (Ekstrand et al. 1994; Frederick et al. 2000) and leads to ligand-independent, constitutive activation of the receptor and – unlike the wild-type receptor – failure to attenuate signaling by receptor downregulation and processing for lysosomal degradation (Huang et al. 1997). The constitutively active EGFRvIII can enhance cell proliferation and has more powerful transforming activity through activation of the PI3K/Akt pathway (Narita et al. 2002). This variant only occurs in GBMs with concurrent wild-type EGFR amplification (Ekstrand et al. 1992). In a further 15% of EGFR mutations, there is a deletion of the codons 521–603 in the region just proximal to the hydrophobic transmembrane domain. Other types of mutations (i.e., missense mutations or insertions) also occur, but at low frequency (3–4%) (Barker et al. 2001; Hackel et al. 1999; Lal et al. 2002; Nishikawa et al. 1994).

Amplification and mutation of the EGFR with subsequent alteration of EGFR expression has multiple effects on tumorigenicity by increasing proliferative capacity and reducing apoptosis (Nishikawa et al. 1994). In addition, tumor cells containing EGFRvIII become more invasive with increased cell motility and infiltrative capacity into the brain via upregulation of effectors of tumor invasion, such as matrix metalloproteinases (MMPs) and serine proteases (Lal et al. 2002). Finally, EGFR mutations confer resistance to radiation and chemotherapy. The

activation of the EGFR/MAPK cascade appears to be a cytoprotective response by tumor cells that abrogates the cytotoxic effect of radiation. This may also apply to tumors in vivo, since patients with an EGFR-positive GBM have a poorer radiographic response (Barker et al. 2001). Furthermore, immunohistochemistry revealed matrix metalloproteinase-9 (MMP-9) in 69% of primary GBM but only 14% of secondary GBM. Active MMP-9 expression was strongly correlated with EGFRvIII expression (Choe et al. 2002).

### 1.2.2 PDGF/PDGFR Signaling Pathway

The *platelet-derived growth factor* (PDGF) has two well-characterized chains (A and B) and two chains (C and D) that were discovered recently (Aaronson 1991; Lokker et al. 2002; Maher et al. 2001). Active PDGF consists of disulfide-bonded homo- and heterodimers of the various chains (AA, AB, BB, CC and DD). There are also two isoforms of the *platelet-derived growth factor receptor* (PDGFR  $\alpha$  and  $\beta$ ), which function as homo- or heterodimers ( $\alpha\alpha$ ,  $\alpha\beta$  and  $\beta\beta$ ) (Hubbard and Till 2000; Maher et al. 2001; Westermarck et al. 1995).

PDGF and PDGFR have been implicated to play critical oncogenetic roles in the transformation process of glial tumors including tumor neovascularization (Guha et al. 1995; Lokker et al. 2002; Maher et al. 2001; Westermarck et al. 1995). The most abundantly expressed isoform is PDGF-AA with lesser amounts of PDGF-AB and BB (Lokker et al. 2002). PDGF-A/-B and PDGFR- $\alpha$  have both been found to be overexpressed in glial tumors of all grades, and increased expression is correlated with higher tumor grade (Di Rocco et al. 1998; Hermanson et al. 1992), whereas PDGFR- $\beta$  expression was generally very low. The coexpression of both PDGF and PDGFR- $\alpha$  in the tumor cells suggests both autocrine and paracrine forms of cell proliferation stimulation (MAPK and PI3K pathways), including paracrine effects on associated blood vessels (Shapiro 2001). The development of autocrine stimulatory loops is an early oncogenetic transforming event (Guha et al. 1995; Hermanson et al. 1992; Lokker et al. 2002; Westermarck et al. 1995). Finally, a coexpression of PDGF-B and PDGFR- $\beta$  was frequently noted in endothelial cells of hyperplastic capillaries in high-grade tumors (Maxwell et al. 1990; Plate et al. 1992a; Shapiro 2001). PDGF receptor and ligand overexpression tend to be associated with TP53 tumor suppressor loss, characteristic of secondary GBMs (Shapiro 2001).

### 1.2.3 TGF/TGF-R Signaling Pathway

*Transforming growth factor* (TGF)  $\alpha$  is a polypeptide of the EGF family of peptide growth factors with 30–40% identical sequence homology to EGF and functions as a monomeric ligand of the EGFR (Aaronson 1991; Maher et al. 2001; von Deimling et al. 1995). In high-grade glioma cells, frequent coexpression of TGF- $\alpha$  and EGFR is present, suggesting auto- and paracrine growth stimulation similar to EGF/EGFR and PDGF/PDGFR. Expression of TGF- $\alpha$  is significantly less frequent in low grade

gliomas (Aaronson 1991; El-Obeid et al. 1997; Maher et al. 2001; Samuels et al. 1989; Tang et al. 1997).

*Transforming growth factor* (TGF)  $\beta$  is a dimeric polypeptide growth factor with three isoforms (TGF- $\beta$ -1/-2/-3), each encoded by a separate gene. After secretion, the majority of TGF- $\beta$  is stored as a complex of TGF- $\beta$ , the TGF- $\beta$  propeptide and TGF- $\beta$  binding protein. TGF- $\beta$  is released from the complex by thrombospondin-1 (TSP-1) alteration of the conformation of the TGF- $\beta$  binding protein or through plasmin-mediated cleavage of the complex. TGF- $\beta$  binds to the high-affinity TGF- $\beta$  receptor (TGF- $\beta$ R), which also has three subtypes (TGF- $\beta$ R1/II/III) (Blobe et al. 2000; de Caestecker et al. 2000; Massague 1998). Only TGF- $\beta$ R1 and TGF- $\beta$ RII contain serine–threonine protein kinases in their intracellular domains; however, TGF- $\beta$ RIII is the most frequent subtype. TGF- $\beta$ RIII binds TGF- $\beta$  and transfers the ligand to TGF- $\beta$ RII, but TGF- $\beta$  can also bind directly to TGF- $\beta$ RII. Once TGF- $\beta$ RII is activated by the ligand, it recruits, binds and transphosphorylates TGF- $\beta$ R1, resulting in TGF- $\beta$ R1 serine–threonine kinase activity and phosphorylation of several transcription factors known as Smads. Smad-complexes interact with other transcription factors in the nucleus to regulate the transcription of numerous TGF- $\beta$ -responsive genes (Blobe et al. 2000).

In normal epithelial, endothelial, glial and hematopoietic cells, TGF- $\beta$  is a potent inhibitor of cell proliferation (cell cycle arrest in G1 phase) and promoter of differentiation and apoptosis. In contrast, in malignant cells, oncogenetic changes of the TGF- $\beta$  signaling pathway are able to abrogate TGF- $\beta$ -mediated growth inhibition resulting in uncontrolled proliferation. Mutations in the TGF- $\beta$ R1, TGF- $\beta$ RII or Smad2 and Smad4 genes cause loss or reduced expression of TGF- $\beta$  receptors. Once the transformed TGF- $\beta$  resistant phenotype has been established, it is common for tumor cells to increase production and secretion of TGF- $\beta$ , which stimulates tumor cells to a more aggressive and invasive phenotype with increased motility, immunosuppression, angiogenesis, deposition of extracellular matrix and alteration of binding to adhesion molecules (Jennings and Pietenpol 1998; Platten et al. 2001).

Malignant gliomas are known to release TGF- $\beta$  and to express all three TGF- $\beta$  receptor subtypes, suggesting auto- and paracrine stimulatory loops (Jennings and Pietenpol 1998; Platten et al. 2001). In high-grade gliomas, TGF- $\beta$ 2, TGF- $\beta$ R1 and TGF- $\beta$ RII are expressed at a significantly higher level than in low-grade tumors (Jennings and Pietenpol 1998; Platten et al. 2001). In most in vitro studies, the TGF- $\beta$  receptors that are expressed appear to be functional, suggesting that downstream abnormalities (e.g., reduced expression of Smad2, 3 or 4) cause the unresponsiveness of these malignant cells to the normal inhibitory effects of TGF- $\beta$  (Isoe et al. 1998). The secretion of TGF- $\beta$ 2 by glioma cells inhibits the activity of infiltrating immune cells and contributes to the escape of neoplastic astrocytes from immune surveillance (Maxwell et al. 1990). TGF- $\beta$ 2 also induces expression of matrix metalloproteinases (MMP-2) and reduces the expression of tissue inhibitor of MMP-2 (TIMP-2), thereby promoting glioma invasive capacity (Wick et al. 2001). A conversion of TGF- $\beta$  from an autocrine inhibitor to a stimulatory mitogen occurs late in the oncogenesis to a malignant glioma (Maxwell et al. 1990; Wick et al. 2001).

### 1.2.4 FGF/FGFR Signaling Pathway

The *fibroblast growth factor* (FGF) family comprises 19 different polypeptides that are involved in mitogenesis, differentiation and angiogenesis (Dunn et al. 2000; Klagsbrun 1989). In gliomas, FGF1 (acidic FGF) and FGF2 (basic FGF) are the best-characterized family members. FGFs exert their biological activity through binding and stimulation of four specific subtype RTKs (FGFR1–4). Compared to normal brain, both FGF and FGFR are highly expressed in human gliomas (Dunn et al. 2000; Stefanik et al. 1991). Expression of FGF and several subtypes of FGFR in tumor and endothelial cells have been shown to correlate with tumor grade in gliomas (Dunn et al. 2000; Stefanik et al. 1991). In addition, nuclear accumulation of FGF2 has been associated with more malignant gliomas and confers a poor prognosis (Fukui et al. 2003; Joy et al. 1997). Malignant progression from low to high-grade gliomas appears to involve upregulation of FGFR expression (Yamaguchi et al. 1994). The most significant contribution of FGF and FGFR seems to be the malignant transformation of gliomas to a more aggressive angiogenic phenotype.

### 1.2.5 IGF/IGFR Signaling Pathway

The *insulin growth factor* (IGF) family plays an important role in cell differentiation, proliferation, apoptosis and transformation (Le Roith 1997; Yu and Rohan 2000). Family members consist of insulin and IGF-I/-II. IGF-I (Somatomedine C, mediator of the effects of the human growth hormone). Associated receptors are the insulin receptor as well as IGF-IR and -IIR. IGF-IIR does not have tyrosine kinase activity, binds IGF-II and functions as antagonist to IGF-II biological activity. IGF-I and II are potent mitogenes and apoptosis inhibitors for numerous solid malignancies and are frequently overexpressed (Trojan et al. 2007). In gliomas, IGF-I, -II and IGF-R are strongly expressed, indicating auto- and paracrine stimulatory loops (Trojan et al. 2007).

### 1.2.6 Gas6/Axl Signaling Pathway

The *receptor tyrosine kinase* Axl is characterized by an extracellular domain consisting of two immunoglobulin-like domains in juxtaposition to two fibronectin-type-III domains (Janssen et al. 1991; O'Bryan et al. 1991), typical for cell adhesion molecules of the immunoglobulin superfamily (Stoeckli and Landmesser 1995). The *growth arrest specific gene 6* (Gas6) is the natural ligand of Axl (Manfioletti et al. 1993; Varnum et al. 1995). Axl/Gas6 signaling has been shown to regulate survival, proliferation and migration in a variety of cells in vitro, including tumor-derived cell lines of epithelial, mesenchymal and hematopoietic origin (Hafizi and Dahlback 2006; O'Bryan et al. 1991). Recently, Axl has been identified to be

overexpressed in the majority of glioma cell lines and was associated with invasive properties of glioma cell lines in a spheroid as well as in a xenograft mouse model (Vajkoczy et al. 2006).

In glioma tissues, Axl and Gas6 are detectable in gliomas of malignancy grades WHO II–IV. Moderate to high Axl protein expression was found in 55% and Gas6 protein expression in 74% of GBM samples. GBM patients with high Axl expression and Axl/Gas6 coexpression showed a significantly shorter time to tumor progression and an association with poorer overall survival. Comparative immunohistochemical studies demonstrated that Axl staining was most pronounced in glioma cells of pseudopalisades and reactive astrocytes. Additionally, Axl/Gas6 coexpression was observed in glioma cells and tumor vessels. In conclusion, these results indicate that inhibition of the Axl/Gas6 signaling pathway may represent a new biologically relevant target for glioma treatment (Hutterer et al. 2008).

### 1.3 Major Signaling Pathways Regulating Tumor-Angiogenesis

#### 1.3.1 VEGF/VEGFR Signaling Pathway

Aberrant extensive *vascular endothelial growth factor* (VEGF) and *vascular endothelial growth factor receptor* (VEGFR) expression in glioma and vascular cells play significant roles in the pathophysiology of glioma growth, and VEGF may be the major endothelial mitogen for these tumors (Dvorak 2002; Machein and Plate 2000; Plate 1999; Wesseling et al. 1997).

VEGF (known as VEGF-A) is a major permeability and proangiogenic factor that is partly responsible for the loss of the blood–brain barrier (BBB) during tumor growth. VEGF is a dimeric glycosylated protein with structural homology to PDGF and produced in at least four isoforms. Three isoforms (VEGF<sub>121</sub>, VEGF<sub>165</sub> and VEGF<sub>189</sub>) have been demonstrated in GBM, with VEGF<sub>165</sub> as the most common (Machein et al. 1999).

Hypoxia (low pO<sub>2</sub> levels) and acidosis (low pH levels) have been shown to independently activate the VEGF promoter and transcription in glioma cells, indicating that VEGF is upregulated by hypoxia and acidosis via different mechanisms (Fukumura et al. 2001; Helmlinger et al. 1997). Hypoxia leads to stabilization of VEGF mRNA and induces expression of HIF-1, which upregulates expression of VEGF through binding to hypoxia-inducible factor (HIF-1) consensus sequence in the 5' flanking region of the VEGF gene (promoter).

VEGF is produced by malignant glioma cells, which are distributed both centrally in and peripherally to the malignant glioma (Johansson et al. 2002; Machein et al. 1999). Centrally, VEGF is typically expressed in malignant cells juxtaposed to areas of necrosis-forming pseudopalisades. Pseudopalisading cells are cell formations composed by a wave of actively migrating tumor cells, moving away from a central hypoxic region. The cell activation for a migrating phenotype results from an increased metabolic demand (low pO<sub>2</sub> and pH levels) due to a high tumor cell

proliferation rate and/or from vasoocclusion due to intravascular thrombosis (Brat et al. 2004; Brat and Van Meir 2004). Peripherally, VEGF can be demonstrated in malignant cells infiltrating the normal brain and in vascular cells of newly developing blood vessels (Brat et al. 2004; Brat and Van Meir 2004). High VEGF levels are also detectable in cyst fluids from primary and metastatic brain tumors cerebrospinal fluid (CSF) of meningeosis carcinomatosa patients, indicating that VEGF is produced at the tumor site and abundantly released into the cyst fluid and CSF (Stockhammer et al. 2000a, b).

VEGF expression in glioma cells is regulated by multiple oncogenes (e.g., growth factors such as bFGF, PDGF, and EGF, and its growth factor receptors), tumor-suppressor genes (e.g., Ras, Src, inactivation of the p53 gene or Hippel-Lindau gene), hormones, cytokines and various intracellular signaling molecules (e.g., nitric oxide and mitogen-activated protein kinases) (Carmeliet and Jain 2000; Dvorak 2002; Ferrara 2004; Maity et al. 2000; Tsai et al. 1995). VEGF can also be released from the extracellular matrix (Carmeliet and Jain 2000; Fukumura et al. 1998).

Intratumoral levels of VEGF and its receptor VEGFR-2 correlate with the histological grade of gliomas (Samoto et al. 1995). Furthermore, VEGF is predominantly expressed in primary GMBs compared to secondary GBM (Godard et al. 2003). Because VEGF is induced by HIF-1, this difference may explain the higher frequency and larger extent of necrosis in primary GBM. In contrast, PDGF-AB shows significantly higher expression levels in secondary glioblastomas (Huang et al. 1997). This suggests that effective antiangiogenic therapy probably requires targeting multiple angiogenic pathways that differ significantly between primary and secondary glioblastomas (Karcher et al. 2006). Finally, radiation stimulates VEGF expression in cultured glioma cells (Gorski et al. 1999), and multiple doses of radiation had a greater effect than single doses (Gorski et al. 1999; Park et al. 2001).

VEGF binds to glioma and vascular cells via interaction with high-affinity RTKs, such as VEGFR-1 (Flt-1, fms-like tyrosine kinase), VEGFR-2 (Flk-1/KDR, fetal liver kinase 1, murine homologue of human kinase insert domain-containing receptor) and VEGFR-3 (flt-4). VEGFR-1 and its soluble isoform (sflt-1) are thought to act as negative regulators of vascular growth by modulating VEGF availability via high affinity binding (Gagnon et al. 2000). VEGFR-2 is the physiologically active receptor isoform and is phosphorylated in response to VEGF binding with subsequent induction of endothelial cell proliferation. Activation of VEGFR-2 induces a cascade of internal signal transduction mediators, such as PI3K-Akt-PTEN, Ras-Raf1-MEK-MAPK, Ras-MEKK-SEK1-JNK-AP1 and PKC-PIP2-DAG-ELK1 pathways. Finally, VEGFR-3 is highly expressed in adult lymphatic endothelium and in tumor vasculature (Hatva et al. 1995; Plate et al. 1994). VEGF receptors are extensively upregulated in the developing microvessels of gliomas. VEGFR-1 and -2 are expressed in endothelial cells of the tumor vasculature as well as in normal brain vasculature adjacent to the glioma, but not in the established vessels of the normal brain distant to the tumor (Hatva et al. 1995; Plate et al. 1994).

The most important intracellular signaling pathway in regulation of tumor angiogenesis via activated endothelial cells is the PI3K-Akt-PTEN pathway through controlling the expression of VEGF, HIF-1 and TSP-1 (Blancher et al. 2001; Jiang et al. 2001; Wen et al. 2001; Zagzag et al. 2000). Overactivity of the PI3K/Akt pathway, either by excessive stimulation by upstream growth factors or by loss of PTEN function, directly leads to upregulation of VEGF expression and secondarily through increased expression of HIF-1 (Blancher et al. 2001; Jiang et al. 2001; Wen et al. 2001; Zagzag et al. 2000). HIF-1 is a heterodimeric transcriptional activator composed of HIF-1a and HIF-1b subunits and is highly expressed in ECs and tumor cells of GBMs induced by hypoxia and can induce expression of VEGF and PDGF (Blancher et al. 2001; Jiang et al. 2001; Wen et al. 2001; Zagzag et al. 2000). TSP-1 is a negative regulator of angiogenesis that is downregulated in cells with loss of PTEN function and overactivity of the PI3K/Akt signaling pathway.

### 1.3.2 Angiopoietins

Another important component regulating angiogenesis in normal and malignant tissues is the angiopoietin pathway (Davis et al. 1996; Maisonpierre et al. 1997; Plate 1999). Two angiopoietin family members have been identified, *Angiopoietin 1* (Ang-1) and *Angiopoietin 2* (Ang-2). Ang-1 is the ligand for the physiologically active angiopoietin receptor TIE2/TEK. Ang-2 is a biological antagonist of Ang-1-mediated TIE2 activation and regulates Ang-mediated angiogenesis. Ang-1 and TIE2 are expressed and active in human glioma cell lines and transgenic mouse astrocytoma models and correlate with the grade of astrocytoma with the highest expression noted in GBM (Ding et al. 2001b; Stratmann et al. 1998). The Ang-TIE2 receptor signaling pathway is an important early event along the angiogenic pathway (Holash et al. 1999; Zagzag et al. 1999). Ang-2 was identified as an early signal which destabilizes vascular endothelium, and the coincident expression of Ang-2 and VEGF produced neoangiogenesis. Ang-2 expression in the absence of VEGF leads to endothelial cell death and vascular regression (Holash et al. 1999; Zagzag et al. 1999).

### 1.3.3 Integrins

Integrins are involved as regulators of angiogenic and apoptotic processes and in brain tumor cell and astrocyte recognition, adhesion and migration on extracellular matrix (ECM). Integrins expressed on the surface of endothelial cells are important for cell-to-cell signaling and the cell attachment to the ECM by binding to various ECM components such as fibronectin, vitronectin, collagen and fibrinogen. VEGF drives a pathway in which  $\alpha_v\beta_5$  integrin is upregulated, whereas bFGF (basic FGF) drives a pathway mediated by  $\alpha_v\beta_3$  integrin (Friedlander et al. 1995; Gladson 1996;

Hu et al. 2006). Recently, clinical trials with integrin antagonists have been initiated for the treatment of malignant gliomas.

## 1.4 Major Intracellular Signal Transduction Pathways

### 1.4.1 Ras Signal Transduction

Ras is a key intermediate in the signal transduction pathways linking membrane-bound receptor tyrosine kinases to downstream cascades of protein kinase effector molecules (Adjei 2001; Boguski and McCormick 1993; Bollag and McCormick 1991; Rowinsky et al. 1999). Ras signaling is involved in cell growth, differentiation, cytoskeletal organization, membrane trafficking and apoptosis. G-Protein Ras functions as a molecular switch and cycles between an inactive guanosine diphosphate (GDP)-bound form (Ras-GDP) and an active GTP-bound form (Ras-GTP). Before Ras can attach to the inner cell membrane and become active, it has to undergo several posttranslational modification steps at the C-terminus to increase its hydrophobicity. The first and most critical step is a farnesylation catalyzed by the farnesyl transferase (FTase). Current data suggest that activation of the Ras signal pathway in malignant gliomas is due to aberrant expression and overactivity of RTKs upstream of Ras (Ding et al. 2001a, b).

### 1.4.2 PI3K/Akt/PTEN/mTOR Signaling Pathway

The primary event in this pathway is the activation of *phosphatidylinositol-3-kinase* (PI3K), which can occur from activation of numerous GFR (e.g., EGFR, PDGFR, FGFR, IGF-IR, VEGFR), integrin receptors and the Ras-pathway. PI3K activates a cascade of downstream effectors that mediate tumor growth and survival (Aaronson 1991; Hackel et al. 1999; Klagsbrun 1989; Lowy and Willumsen 1993; Maher et al. 2001; Martin and Blenis 2002; Rameh and Cantley 1999; Shapiro 2001; Vivanco and Sawyers 2002; Wymann and Pirola 1998) by catalyzing the transfer of the G-phosphate of ATP to the head group of phosphatidylinositol (PI) membrane lipids, such as PI, PIP1(4) and PIP2(4,5).

In malignant gliomas, the PI3K/Akt signaling pathway is frequently overactive, which can be partially explained by upstream activation from GFR and Ras (Choe et al. 2003; Sakata et al. 2002). Constitutively increased activity of PI3K and Akt correlates with increased invasiveness of malignant gliomas (Kubiakowski et al. 2001), probably by coexpression with matrix metalloproteinases MMP-2 and -9 (Sonoda et al. 2001).

The PI3K/Akt pathway has several forms of regulation. The most important regulation is the tumor suppressor gene *PTEN* (*phosphatase and tensin homolog*) (Li et al. 1997; Steck et al. 1997). The PTEN gene is located on chromosome 10q23.3 and encodes a cytoplasmatic protein with tyrosine phosphatase activity

(N-terminus) and a region that interacts with the cellular cytoskeleton (Cantley and Neel 1999; Li et al. 1997; Maehama and Dixon 1998; Steck et al. 1997; Tamura et al. 1999). Hemizygous deletion of the wild type allele is frequent in GBM (60–80%) (Vazquez and Sellers 2000). The remaining copy of the gene is then inactivated by frameshift, nonsense or missense mutations. PTEN mutations and loss of function are responsible for the abnormally high levels of the PI3K/Akt signaling pathway (Duerr et al. 1998; Knobbe et al. 2002; Rasheed et al. 1997; Sano et al. 1999; Vazquez and Sellers 2000; Wang et al. 1997). There is a strong predilection for PTEN mutations to occur in high-grade tumors with a reported frequency of 15–40% in primary de novo GBM. Secondary GBM are much less likely to harbor PTEN mutations (<10%, Table 2), and PTEN mutations are rare or absent in low grade astrocytomas and oligodendrogliomas and have an incidence of only 5–10% in AGs. Allelic loss of 10q, mutations of the PTEN gene and alterations of PTEN expression have a negative impact on the prognosis of patients with gliomas (Ermoian et al. 2002; Knobbe et al. 2002; Sasaki et al. 2001; Smith et al. 2001). Patients with AODs that have allelic loss of chromosome 10q have a poorer response to chemotherapy and a significantly shorter survival time than patients with an intact 10q (Sasaki et al. 2001).

*Mammalian target of rapamycin* (mTOR) is a signal transduction mediator with serine/threonine protein kinase activity, intimately linked to the PI3K/Akt signaling pathway and the regulation of protein synthesis and cell growth (Dennis et al. 1999; Martin and Blenis 2002; Schmelzle and Hall 2000; Sekulic et al. 2000). mTOR is activated in response to growth factor signals through the PI3K/Akt pathway with subsequent mTOR serine phosphorylation (Dennis et al. 1999; Inoki et al. 2002; Martin and Blenis 2002; Schmelzle and Hall 2000; Sekulic et al. 2000; Tee et al. 2002).

## 2 Neoangiogenesis in Malignant Gliomas

Neoangiogenesis in solid tumors is a tightly controlled process that involves growth and maintenance of blood vessels (Folkman 1995). An equilibrium exists between proangiogenic (e.g., VEGF, PDGF, bFGF, TGF- $\beta$ , angiopoietins) and inhibitory factors (e.g., thrombospondin TSP-1, glioma-derived angiogenesis inhibitory factor, tissue inhibitor of MMP-1, angiostatin and endostatin) (Folkman 1995; Hanahan and Folkman 1996). These factors interact with specific receptors (e.g., VEGFR-1/2/3, TIE-1/2) on vascular cells, including endothelial cells (EC), vascular smooth muscle cells (VSMC) and pericytes (PC) (Plate 1999), surrounding extracellular matrix (ECM)-associated adhesion molecules (e.g., integrins) and matrix metalloproteinases (MMPs) (Couldwell et al. 1992; Rutka et al. 1988; Uhm et al. 1997).

Normal brain vasculature is highly specialized and composed of endothelial cells, pericytes and astrocytes. These cells form and maintain the blood brain barrier (BBB) which restricts the exchange of molecules between the intracerebral and extracerebral circulatory systems. First, tight junctions between endothelial

cells prevent transcapillary movement of hydrophilic molecules varying in size from proteins to ions entering the brain parenchyma. Secondly, there are no detectable transendothelial pathways in brain endothelial cells. Finally, in the brain endothelium, several active, receptor-mediated transport proteins (e.g., P-glycoprotein/multidrug resistance proteins) exclude exogenous compounds from the brain parenchyma and, consequently, contribute to drug resistance (Deeken and Loscher 2007; Neuwelt et al. 2008).

## 2.1 Cellular Mechanisms

After an initial clonal expansion of a primary brain tumor beyond 1–2 mm in diameter within the brain parenchyma (approximately a million cells), the tumor must acquire an angiogenic phenotype by cooption followed by angiogenesis and vasculogenesis to enlarge. Initial brain tumor growth occurs by *cooption of normal brain capillaries* (Holash et al. 1999; Leenders et al. 2002). During tumor growth, cancer cells migrate along blood vessels providing the invading tumor cells with oxygen and nutrients. However, the tumor cells compress and destabilize the tumor vasculature. Stromal-cell-derived factor 1 (SDF1) contributes to tumor cell invasion and to the ability to coopt new vessels, whereas angiopoietin-1 maintains vessel integrity. Angiopoietin 2, produced by tumor or endothelial cells, can bind to the TIE2 receptor on endothelial cells and destabilize the vessels (Holash et al. 1999; Stratmann et al. 1998). This process leads to vessel regression and reduced perfusion resulting in hypoxia and even tumor cell death (necrosis and apoptosis) (Holash et al. 1999; Padera et al. 2004). Hypoxia and mutations in cancer cells induce the secretion of growth factors (e.g., VEGF, bFGF, IL8 and SDF1) that recruit new blood vessels through *angiogenesis* (Carmeliet 2005; Carmeliet and Jain 2000; Ferrara et al. 2004; Yancopoulos et al. 2000). In addition, PDGF can upregulate VEGF and modify autocrine/paracrine effects on endothelial and perivascular cells. Bone-marrow-derived cells (e.g., endothelial progenitor cells) can be recruited to increase the tumor vascular supply by direct incorporation into functional vasculature (Duda et al. 2006).

## 2.2 Molecular Mechanisms

Angiogenesis in primary brain tumors is mainly driven by *VEGF signaling through its endothelial receptor VEGFR-2*. High VEGF-concentrations, up to a 50-fold increase, have been detected in malignant gliomas (Fischer et al. 2005; Holash et al. 1999; Millauer et al. 1994; Plate et al. 1992a, b, Stockhammer et al. 2000a, b). VEGFR-2 is the main mediator of several physiological and pathological effects of VEGF on endothelial cells, including survival, proliferation, migration and vascular permeability (Carmeliet and Jain 2000; Dvorak 2002; Ferrara 2004). As an initial

VEGF response, vessels dilate, become leaky and – together with angiopoietin-2 and several proteinases – VEGF mediates the dissolution of the existing basement membrane and interstitial matrix. Furthermore, VEGF (through an interaction with VE-cadherin) is a critical survival factor of endothelial cells (Carmeliet et al. 1999).

Numerous other molecules stimulate endothelial cell proliferation, survival, migration and assembly into vascular networks, such as angiopoietins (Ang), basic fibroblast growth factor (bFGF), granulocyte macrophage-colony stimulating factor (GM-CSF), insulin-like growth factor 1 (IGF1), stroma derived growth factor (SDF, CXCL12), interleukin 8 (IL8), stem-cell factor (SCF) and delta-like 4 ligand (DLL4). *Angiopoietins* have a major role in coopting tumor vessels. Ang-1 acts as a survival factor of endothelial cells and is important in vessel cooption (Carmeliet and Jain 2000; Reiss et al. 2005; Yancopoulos et al. 2000). Ang-2 is a complex regulator of vascular remodeling that plays a role in vasculature destabilizing and sprouting as well as in vessel regression during angiogenesis (Ding et al. 2001a, b; Holash et al. 1999; Hu et al. 2006; Maisonpierre et al. 1997). Ang-2 also stimulates MMP-2 expression leading to glioma cell invasion by interacting with  $\alpha(v)\beta(1)$  integrin in Tie2-deficient human glioma cells (Hu et al. 2006). *bFGF* is a potent mitogen for endothelial cells and has been implicated in GBM angiogenesis (Carmeliet and Jain 2000; Stan et al. 1995). VEGF, angiopoietins, bFGF, *GM-CSF*, *IGF1* and *SDF1* have been implicated in systemic effects through mobilization of endothelial precursors by mediating chemokinesis, chemotaxis and tissue retention (Carmeliet and Jain 2000; Grunewald et al. 2006; Hattori et al. 2001; Yancopoulos et al. 2000). In addition, the chemokine SDF1 and its receptor CXCR4 are expressed in endothelial and glioma cells. Both are typically colocalized in glioblastoma cells, mainly in regions of pseudopalisades surrounding necrosis and microcystic degeneration, and the levels of CXCR4 correlate directly with those of HIF1 $\alpha$  in hypoxic regions of tumors (Bajetto et al. 2007; Zagzag et al. 2000). *IL8* is a proangiogenic chemokine expressed and secreted at high levels in human gliomas (Salmaggi et al. 2003). *IL8* levels correlate with histological grade of gliomas (Melder et al. 1996). The highest expression was found in GBMs located in pseudopalisading cells around areas of necrosis suggesting that hypoxia may stimulate its expression (Brat et al. 2005; Shweiki et al. 1992). *SCF* is expressed in glioma cells and may have a role in tumor-induced angiogenesis in the brain (Sun et al. 2006). Many multitargeted tyrosine kinase inhibitors inhibit SCF signaling through blocking its receptor c-Kit. The *Delta-like 4 (DLL4)-mediated Notch signaling* represents another key pathway essential for vascular development in brain tumors (Gridley 2007). DLL4 is downstream of VEGF signaling and its activation triggers a negative feedback that restrains the effects of VEGF. Attenuation of DLL4/Notch signaling results in chaotic vascular network with excessive branching and sprouting. In preclinical studies, blocking of DLL4/Notch signaling is associated with a paradoxical increase in tumor vessel density, yet causes marked growth inhibition due to functionally defective vasculature. DLL4 blockade holds promise as an additional strategy for angiogenesis-based cancer therapy, especially when resistance to and/or escape from existing therapies evolve (Leslie et al. 2007; Ridgway et al. 2006; Williams et al. 2006).

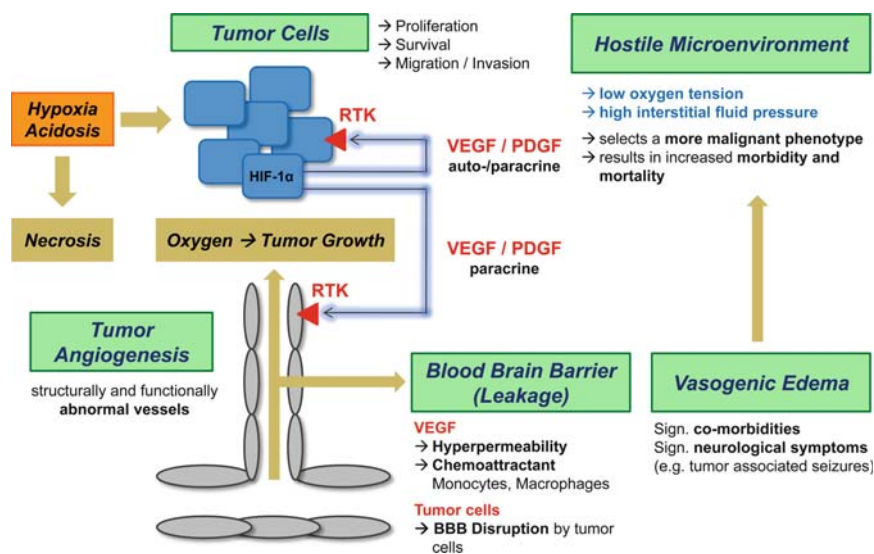
Other important stimuli are an overactivity of major *growth factor signaling pathways* (e.g., EGF/EGFR, PDGF/PDGFR, FGF/FGFR, IGF/IGF-R and TGF- $\beta$ /TGF- $\beta$ R) and loss of certain *tumor suppressor genes* (e.g., PTEN) (Dunn et al. 2000; Maher et al. 2001; Platten et al. 2001; Shapiro 2001; Takahashi et al. 1992). Increased levels of tumor-derived growth factors and their receptors can stimulate increased secretion of VEGF and initiate endothelial cell activity. Endothelial cell activity including spreading and migration in response to growth factor signaling is mediated by *cell-matrix receptors* (e.g., integrins  $\alpha_v\beta_5$ ,  $\alpha_v\beta_3$  and  $\alpha_5\beta_1$ ), and upregulate *critical proteases* from endothelial cells to remodel the surrounding extracellular matrix molecules and permit endothelial cell migration (Hood and Cheresch 2002). For example, PDGFR isoforms (A–D) and their receptors (PDGFR- $\alpha$ /- $\beta$ ) (Dunn et al. 2000) are not only expressed in perivascular cells but also in brain tumor endothelial cells (Batchelor et al. 2007). PDGF-B can upregulate VEGF and exert autocrine effects on endothelial and perivascular cells (Fredriksson et al. 2004; Hermansson et al. 1988).

### 2.3 *Structural Abnormalities of Brain Tumor Blood Vessels and Blood Brain Barrier*

In malignant gliomas the tumor vasculature and the blood brain barrier (BBB) are structurally and functionally abnormal, which contributes to a hostile microenvironment with low oxygen tension and high interstitial fluid pressure. Such a microenvironment selects for a more malignant phenotype resulting in increased morbidity and mortality (Fidler 2001; Fukumura et al. 2001; Hobbs et al. 1998; Izumi et al. 2002; Yuan et al. 1994) (Fig. 3). Furthermore, brain tumors exhibit other neuropathological vascular features, represented by increased vascularity with:

- Distinct endothelial cell proliferation with prominent swollen endothelial cells
- Disorganized, poorly connected, highly permeable tumor vessels including endothelial wall abnormalities, pericyte coverage and basal membrane swelling, resembling glomeruloid tufts (consisting of multilayered, mitotically active endothelial cells and perivascular cells), garland-like, clustered or bizarre vessel structures
- Tumor vessels with significantly larger diameters and thicker basement membranes than those of the normal brain and
- Necrotic areas with characteristically surrounding pseudopallisades resulting in regions of hypoxia (vasoocclusion through thrombosis, high proliferation rate)

(Birner et al. 2003; Bullitt et al. 2005; Fidler 2001; Guo et al. 2003; Hobbs et al. 1998; Kleihues and Sobin 2000; Plate and Mennel 1995; Valk et al. 1992; Winkler et al. 2004; Yuan et al. 1994; Zagzag et al. 1999)



**Fig. 3** Principles of tumor growth, tumor angiogenesis and vasogenic tumor edema via autocrine and paracrine VEGF/PDGF signaling

Distinct angiogenic subtypes are diagnostic features in brain tumors, especially in GBMs. In primary GBM patients such morphological vascular abnormalities influence the clinical outcome and are associated with variable expression of angiogenic proteins, relevant for antiangiogenic therapy approaches (Birner et al. 2003). Furthermore, for discrimination between low- and high-grade gliomas angiogenic subtypes and the degree of vascularity are major differentiating features (Plate 1999; Wesseling et al. 1997). In neuropathological studies, astrocytic glioma patients with the highest microvessel density have the most malignant tumors and the shortest survival times, and in oligodendroglioma patients, VEGF expression strongly correlates with neovascularization and tumor progression (Leon et al. 1996; Varlet et al. 2000).

An abnormal vascular structure causes an abnormal vascular function, represented by the loss of selectivity of permeability in brain tumors and generally elevated vascular permeability. MRI studies of patients showed a difference of as much as 20-fold between normal brain permeability and glioma vessel permeability. However, the loss of the BBB is temporally and spatially heterogeneous, depending on the tumor type (e.g., malignancy grade or primary vs secondary metastatic brain tumors), tumor location and even from one day to another (Jain 1998; Jain et al. 2002; Monsky et al. 1999).

Focal BBB leaks contribute to nonuniform blood flow and heterogeneous delivery of oxygen and blood-borne drugs (Jain et al. 2007). This defect results in a significantly reduced red blood cell (RBC) velocity that is independent of vessel diameter (Yuan et al. 1994). In vivo microscopy of a glioblastoma xenograft model showed an activation of the vascular endothelial growth factor (VEGF) promoter,

which correlated with the tissue oxygen level ( $pO_2$ ) and pH level.  $pO_2$  and pH profiles around tumor vessels demonstrate the presence of hypoxia and low pH.

## 2.4 Tumor Edema in Malignant Gliomas

Depended on the tumor location, malignant glioma patients suffer significant morbidity caused by the peritumoral vasogenic edema. The mechanisms of BBB disruption and vasogenic brain edema formation are not completely understood. A potent inducer of vascular hyperpermeability is VEGF. Vascular hyperpermeability results in accumulation of fluid and plasma proteins within the tumor, leading to increased interstitial fluid pressure (IFP) (Jain et al. 2007). Because the brain lacks a lymphatic system, interstitial fluid seeps from the glioma with high IFP into the surrounding brain tissue until the CSF pressure becomes equal to the tumor IFP. The edema tends to extend along white-matter tracts rather than in the more closely packed gray matter (Weissman 1988). In addition, VEGF is known to be a chemoattractant for monocytes/macrophages, which have also been implicated in vasogenic edema pathogenesis (Weissman 1988) (Fig. 3).

Receptor tyrosine kinase (RTK) activation results from receptor dimerization and autophosphorylation of the catalytic domain tyrosine residues with formation of attachment sites for internal signal transduction molecules (second messengers) that have SH2 domains (Westermarck et al. 1995). Phosphorylation of these second messengers induces complex cascade activity within the cell and downstream pathways become activated.

The figure illustrates the complex and heterogeneous network of interacting intracellular signaling pathways.

## Attendum

### Growth Factor Receptors

Typ I: *EGFR (Her1/c-erbB1)*, *Her2/neu (c-erbB2)*, *Her3 (c-erb3)*; Typ II: *InsulinR*, *Insulin-related R*, *IGF-1R*; Typ III: *PDGF $\alpha$ R*, *PDGF $\beta$ R*, *MCSF-1R*, *c-Kit*; Typ IV: *FGFR1-4*; Typ V: *VEGFR1-3*, *Flt-1/Flk*; Typ VI: *HGFR/Met*; Typ VII: *TrkA-C*; Typ VIII: *Eph*, *Elk*, *Eck*, *Eek*, *Erk*, *Cek4/Mek4*, *Cek5*; Typ IX: *Axl*, *Tyro3*, *Mer*.

### Ras-GDP Pathway

Ras-GDP is rapidly and transiently converted to Ras-GTP in response to activation of various RTKs. After autophosphorylation of the receptor active site, adapter proteins with SH2 domains (e.g., Grb2) bind to the receptor and recruit Ras

activator proteins (e.g., Sos-1). Ras activator proteins function as Ras guanine-nucleotide exchange factors (GEFs) by exchange of GDP for GTP, thereby activating Ras. The GTPase activator protein (GAP) promotes hydrolysis of Ras-bound GTP to GDP, converting Ras to its inactive form and inhibiting further signal transduction. Ras-GTP stimulates several downstream effectors, including Raf1 (Raf1-MEK-MAPK-AP1), Rac (Rac-Rho), MEKK (MEKK-SEK1-JNK-AP1), PI3K (PI3K-Akt-P70S6K) and PLC (PLC-DAG-PKC).

## PI3K Pathway

*PI3Ks* are heterodimers composed of a regulatory subunit (p55 or p85) and a catalytic subunit (p110). The regulatory subunit contains regions including a Src homology (SH)-3 domain and two SH2 domains. The SH3 domain allows binding to proline-rich regions of various proteins, while the SH2 domains allow binding to phosphotyrosine residues of other regulatory and signaling molecules. Interaction of the SH2 domains with phosphotyrosine increases lipid kinase activity of the catalytic subunit p110. The p110 subunit contains a kinase domain and interaction sites for the p85 subunit and Ras. A favored substrate for PI3Ks is PIP2(4,5) with the production of PIP3(3,4,5) after phosphorylation. Other targets of PI3K include Rac, p70S6K and certain isoforms of PKC (Martin and Blenis 2002; Rameh and Cantley 1999; Vivanco and Sawyers 2002; Wymann and Pirola 1998).

PIP3 recruits the *serine/threonine kinase Akt* to the juxtamembrane region by binding a lipid head group directly to the pleckstrin homology (PH) domain in the Akt N-terminal segment (Andjelkovic et al. 1997). Simultaneously, PIP3 activates the membrane-associated kinases PIP3-dependent kinases (PDK)-1 and 2. Once Akt is associated with the membrane and bound to PIP3, a conformational change occurs and allows phosphorylation of the threonine position of the catalytic domain by PDK1. While Akt is still in close proximity to the membrane, PDK2 phosphorylates a serine of the hydrophobic C-terminal tail. Phosphorylation of both sites is required for full activation of Akt and must occur before Akt can detach from the membrane and interact with cytosolic or nucleic downstream effectors (Brunet et al. 1999; Cardone et al. 1998; Datta et al. 1999; Vivanco and Sawyers 2002).

*PTEN* can dephosphorylate tyrosine-, serine- and threonine phosphorylated peptides and has lipid phosphatase activity through dephosphorylation PIP3. In addition, there are lipid-binding C2 domains and an SH2 binding site (Vazquez et al. 2001; Vazquez et al. 2000). Phosphorylation of the PTEN C-terminal tail causes a conformational change that suppresses PTEN activity through inhibition of its ability to be recruited into PTEN-associated complexes. The most important cellular PTEN function appears to be its lipid phosphatase activity, which forms the basis for its designation as a tumor suppressor gene (Cantley and Neel 1999; Maehama and Dixon 1998). PTEN dephosphorylates PIP2 and PIP3 directly antagonizing PI3K activity and reducing the activity of PI3K-dependent downstream effectors.

VEGF/PDGF are secreted by tumor cells and activated associated receptor-tyrosine kinases RTKs (VEGFR, PDGFR) of tumor and vascular cells. VEGF leads to a leakage (hyperpermeability) of the blood brain barrier and vasogenic edema, resulting in significant comorbidities, neurological symptoms and a hostile tumor microenvironment with low oxygen tension and high interstitial fluid pressure. The goal of antiangiogenic treatment approaches is to interrupt the VEGF- and/or PDGF- signaling pathways through binding of VEGF using mABs or blocking the RTKs using SMI-Rs.

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