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REVIEW

Adrian Merlo

Genes and pathways driving glioblastomas in humans and murine disease models

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Abstract Human malignant gliomas arise from neural progenitor cells and/or dedifferentiated astrocytes. By now, they are genetically so well characterized that several murine glioma models have emerged that faithfully reiterate the typical histological features of the disease. In experimental animals, only one or two elements of the growth factor/Ras, PI3K/PTEN/PKB, p53/ARF/HDM2, and p16/Rb/cyclinD/CDK4 pathways are targeted. In human gliomas, many additional genes and pathways are targeted due to a most severe mutator phenotype that leads to the accumulation of countless epigenetic and genetic alterations. Changes that convey a growth advantage are selected for, leading to overgrowth of precursor cell populations with increasingly malignant tumor cell clones. While murine models represent a powerful tool for elucidating the role of genetic pathways, mechanisms of response and resistance to new therapeutic agents might be fundamentally different due to the high degree of genomic instability in the human disease. In fact, little is known about the molecular causes of genomic instability involved in gliomas, except for the rare Turcot's syndrome, O_6-methylguanine-DNA methyltransferase, and the apurinic/apyrimidinic endonuclease Ape-1. Novel approaches that selectively exploit fundamental metabolic differences between tumor and normal cells have to consider these fundamental differences between human disease and presently available, highly sophisticated animal models.

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A. Merlo (✉)
Departments of Surgery and Research,
Neurosurgery and Molecular Neuro-Oncology Laboratory,
University Hospitals,
Spitalstrasse 21, 4031 Basel, Switzerland
e-mail: amerlo@uhbs.ch
Tel.: +41-61-2657456
Fax: +41-61-2657138

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The therapeutic dilemma

Many attempts to treat human gliomas, including brain tumor surgery introduced some 100 years ago, have had little effect on the prognosis of this disease which is basically characterized by two biological factors: unscheduled cell proliferation and insidious infiltration of normal brain tissue. Transformed glial cells stepwise deprive an affected person of critical neuronal functions such as the ability to perceive properly, reflect, and react to exogenous and endogenous stimuli. Some of these deleterious effects are mediated by factors secreted by glioma cells, e.g., glutamate, interfering with neuronal function [116]. Even though surgical interventions taking advantage of sophisticated online imaging and navigation technology allow MRI-documented "complete" resection improving local tumor control, the further course of the disease remains dominated by unchecked tumor cell infiltration. Glioma cells resist apoptotic stimuli from external beam radiotherapy and virtually all chemotherapeutic agents, except for some remarkable responses observed in the less prevalent cases displaying oligodendroglial differentiation. Even so, impressive chemotherapeutic responses tend to be transient. Emerging new therapies based on molecular mechanisms such as blocking epidermal growth factor receptor (EGFR) [3, 116], the target of rapamycin (mTOR) [22, 121], protein kinase B (PKB)/Akt-1 [12], phosphoinositide 3-kinase (PIK-3) [9], mitogen-activated extracellular signal-regulated kinase activating kinase (MEK) [24], or focal adhesion kinase (FAK) [106] should be evaluated by not only their effect on visible tumor nodules but also their potential to target the invisible infiltrative component of the disease. Innovative techniques allowing visualization and quantitation of infiltrating glioma cells are urgently needed for therapeutic targeting and accurate response assessment.

Developmental cues for the cellular origin of gliomagenesis

Embryonic development of the brain

By embryonic day (E) 8, pluripotent stem cells of the ventricular zone driven by fibroblast growth factor (FGF) first produce radial glial cells in the intermediate zone which proliferate and establish scaffolds that act as guides for neuronal and glial progenitors as they migrate from the ventricular zone [39, 46, 77]. By E13, the pluripotent stem cells become progressively more responsive to EGF, self-renew, and give rise to glial restricted progenitors (Fig. 1). By E18, the stem cells in the ventricular zone are responsive only to EGF and produce the glial restricted PDGFR+ and PDGFR− progenitors, which also migrate from the ventricular zone to the intermediate zone, where they undergo oligodendroglial or astrocytic differentiation. Oligodendrocyte progenitors can differentiate into astrocytes, and astrocytes may dedifferentiate into radial glia [122]. Transformation of radial glial cells into astrocytes and vice versa appears to be regulated by the availability of inducing signals rather than by changes in cell potential [46].

Hypothesis 1: gliomas arise from neural stem or glial progenitor cells

The identification, within the adult CNS, of neural stem cells endowed with the capacity for self-renewal and differentiation into mature astrocytes and neurons revealed an unexpected level of plasticity in the adult brain [97]. Consequently, the hypothesis emerged that a transformed neural stem cell or an early glial progenitor could be the culprit of tumorigenesis. These cells are localized predominantly in the subventricular zone and dentate gyrus of the hippocampus; however, their proliferative and migratory potential allows tumor formation to manifest anywhere in the brain [77]. A premalignant cell could either first migrate out of the subventricular zone to the area of the future tumor nidus where it acquires mutations in cancer-promoting and -progressing genes, or a precursor cell suffers genetic damage in the subventricular zone and subsequently starts to migrate in one or several directions. Both scenarios might be operative in gliomagenesis.

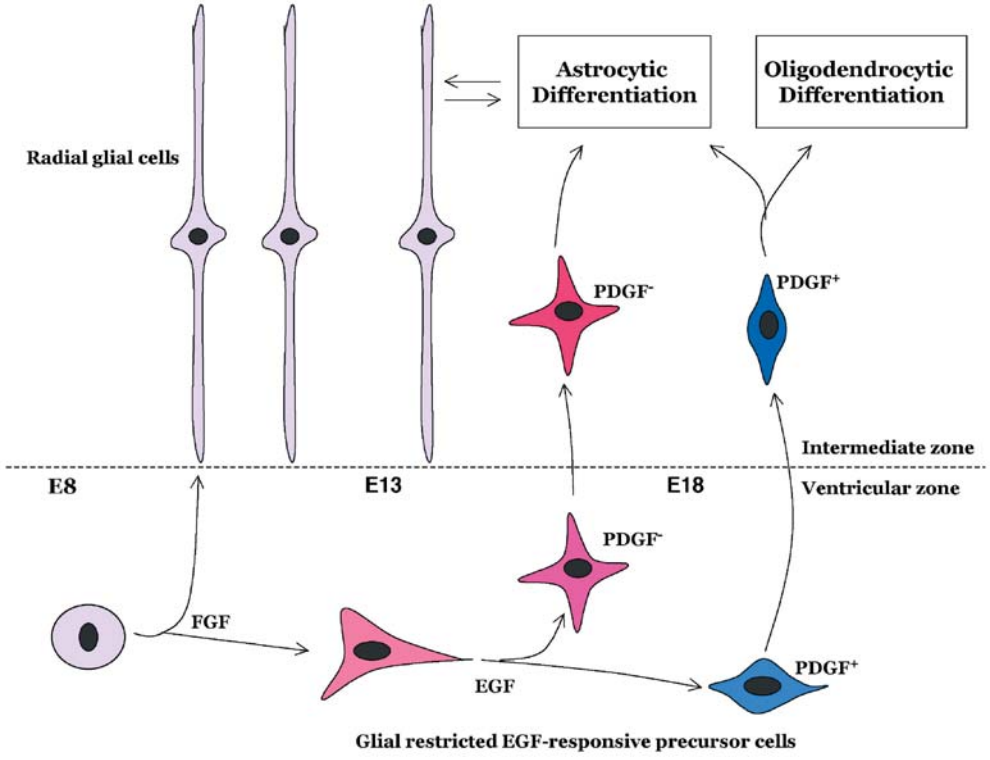

Fig. 1 Embryonic development of glial cells. By embryonic day 8 (E8), FGF-driven pluripotent stem cells of the ventricular zone first produce radial glial cells in the intermediate zone which proliferate and establish scaffolds that guide migration of neuronal and glial progenitors out of the ventricular zone [39, 46, 77]. By E13, the pluripotent stem cells become progressively more EGF responsive and give rise to glial restricted progenitors. By E18, the stem cells in the ventricular zone are responsive only to EGF and produce the glial restricted PDGFR+ and PDGFR− progenitors, which also migrate from the ventricular zone into the intermediate zone, where they undergo oligodendroglial or astrocytic differentiation. Oligodendrocyte progenitors can differentiate into astrocytes, and astrocytes may dedifferentiate into radial glia [122]. Transformation of radial glial cells into astrocytes and vice versa appears to be regulated by the availability of inducing signals rather than by changes in the cell potential [46]

Hypothesis 2: gliomas arise from dedifferentiated astrocytes

Mature astrocytes can dedifferentiate into radial glia, not only at the site of the experimental inoculation of embryonic cells but also at a distance from the site of implantation [46, 113]. This suggests that diffusible factors acting across long distances can change the fate of a cell and that mature astrocytes retain the ability to respond to these factors [77]. Thus, in gliomagenesis, genetic mutations may convert a mature astrocyte into a more immature state of proliferation and migration, possibly facilitated by unspecific stimulation by growth factors which might—in some cases—be transitorily released during traumatic and inflammatory conditions.

Therapeutic implications of neural stem cells

In rodent brain tumor models, neural stem cells have been successfully used to treat experimental gliomas. The gene for interleukin-4 was stably transferred into C57BL6 J mouse primary neural progenitor cells injected into established syngeneic brain glioblastomas, leading to the survival of most tumor-bearing mice. Similar results were obtained by implanting immortalized neural progenitor cells derived from Sprague-Dawley rats into C6 glioblastomas [7]. In another series of experiments, neural stem cells were found to be capable of tracking both tumor mass and infiltrating tumor cells following administration into the contralateral hemisphere or even intravenously [1]. Similar results were reported in an oligodendroglioma transgenic mouse model in which neural stem cells migrated to the tumor after injection at some distance from the tumor [14]. So far, it is not clear whether stem cell migration follows a gradient of topic cues, e.g., growth factors released from tumor cells, or whether stem cell survival is dependent upon the secretion of tumor-derived growth factors, since these cells appear to vanish following injection into normal brain.

Major pathways of gliomagenesis

A large number of genetic alterations have been detected and thoroughly catalogued by now in the great variety of human brain tumors, including the different glioma subtypes [58]. Although more rare than sporadic tumors, familial cancer syndromes provided important clues about the pathophysiology of these neoplasms, illuminating the role of specific genes and associated pathways. This concept has also been tested in animal models that have begun faithfully to reiterate seminal phenotypic features of the human disease. In glioblastomas, several safeguard mechanisms are lost due to primary and/or secondary mutational inactivation, as shown schematically in Fig. 2. An oncogenic challenge to a normal cell—either from within or from an external source—induces cell cycle arrest, DNA repair if possible, or apoptosis. Tumor cells evade these controls by specifically mutating regulators of cell cycle and apoptosis. In addition, an endogenous mutator phenotype leads to the accumulation of countless genetic and epigenetic alterations which offer a rich and

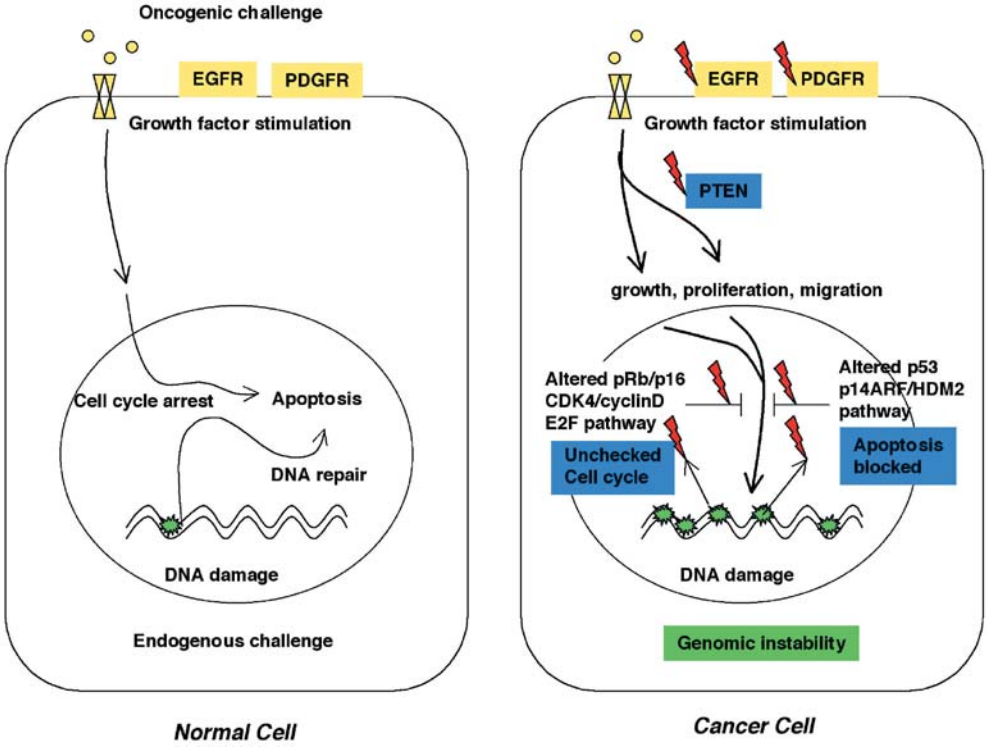

Fig. 2 Different reaction of a normal or a cancer cell to oncogenic stress. Upon an exogenous or endogenous oncogenic challenge, a normal cell induces cell cycle arrest and either repairs damaged DNA or undergoes programmed cell death. A cancer cell cannot elicit efficient cell cycle arrest and apoptosis anymore but manages to survive in spite of accumulated genomic instability

unique repertoire for each cell to select an even more malignant phenotype.

Growth factor signaling

In gliomas, PDGFR and EGFR autocrine signaling are involved in cellular development, proliferation, migration, and vascularization. PDGF and EGF play important roles in glial development: EGF in neural stem cell proliferation and survival, PDGF in glial development [77]. PDGFR are expressed in most types of gliomas [42], while EGFR are mainly expressed in glioblastoma multiforme (GBM) [67]. Of note, PDGFR-A amplification was only found in anaplastic oligodendrogliomas with glioblastomatous features [111]. In highly proliferating tumor cells, PDGFR-A expression was detected as well as PDGFR-B expression, which was also found on endothelial cells [42, 62]. In the adult brain, putative neural stem cells and glial precursor cells expressing α-chains of PDGFR are found in the subventricular zone and scattered throughout the white matter and cerebral cortex [90, 100]. These distinct cell populations could well represent glioma precursor cells. The EGFR signaling pathway is necessary for sustained proliferation and perhaps survival of the neural stem cell compartment. Approximately 40% of GBMs with EGFR amplification also express a variant form called EGFRvIII, ΔEGFR, or del2–7EGFR [30]. This mutant lacks a portion of the extracellular ligand-binding domain as a result of genomic deletions that eliminate exons 2–7 in the EGFR mRNA and which is constitutively autophosphorylated, albeit at a significantly lower level than in ligand-driven, wild-type EGFR phosphorylation [25]. ΔEGFR was aberrantly located in the endoplasmic reticulum with a half-life extended severalfold over that of ligand-activated wild-type receptor, suggesting escape from physiological regulatory mechanisms utilized for attenuating wild-type EGFR [26]. The introduction of this truncated receptor into glioma cells dramatically enhances their tumorigenicity in vivo through both increased cellular proliferation and reduced apoptosis, also conferring resistance against chemotherapeutic drugs such as cisplatin through modulation of Bcl-XL expression [88]. Growth factor signaling mediated by critical ligands like EGF and PDGF activates an intricately complex network of cellular protein domains modulated by G-protein-coupled receptors and second messengers [43] which converge at defined points—crossroads of cell signaling, one of which is Ras—playing a central role in brain tumorigenesis, as recently proven by three murine glioma models [19, 24, 45]. Figure 3 shows schematically most critical pathways or circuits operative in gliomagenesis.

Neurofibromatosis 1 and the critical role of the Ras pathway

The large neurofibromatosis 1 (NF-1) gene encoding neurofibromin is considered a mutational hot spot of the human genome: one out of 3,500 individuals carry a gene mutation which manifests sporadically as de novo mutation in about 50% of cases outside a familial context [19]. In sporadic malignant gliomas, specific mutations affecting Ras have not been detected; however, high levels of Ras guanosine triphosphate (GTP) were documented in astrocytoma cell lines but also in most primary high-grade astrocytomas in one study, suggesting that the Ras effector arm is activated possibly by upstream receptor tyrosine kinase activation, mainly PDGFR and EGFR [37]. The manifestation of optic nerve glioma, astrocytoma, and glioblastoma in NF-1 patients further underscores the critical role of the Ras pathway in brain tumorigenesis. The disturbed differentiation program in mesenchymal and neuroectodermal precursor cells leads to a wide clinical spectrum including cognitive deficits, café-au-lait maculae, Lisch nodules, craniofacial dysplasia, pheochromocytoma, neurofibromas, and myeloid leukemia.

The NF-1 gene negatively regulates Ras as an exchange factor converting Ras-GTP to Ras-GDP by its GTPase-activating (Ras-GAP) domain [19]. RAS-GTP operates downstream of growth factor receptors at a major signal transduction crossroad, translating extrinsic messages into the Raf-MEK-ERK and into either the PI3K-PKB or the PI3K-Rac-Rho pathway. These signaling networks are of prime importance in the regulation of cell survival and migration. The PI3K-Rac-Rho pathway is involved in cell motility and negatively regulated by merlin, the neurofibromatosis type II (NF-2) tumor suppressor that predisposes humans and mice to tumor development and links the cytoskeleton to the membrane [103]. Sustained Raf/MEK/ERK signaling represents an oncogenic challenge that induces a growth arrest and senescence phenotype mediated by $p16^{INK4A}$ and $p14^{ARF}$ in normal astrocytes [28]. In INK4A/ARF-deficient glioblastoma cells, a $p21^{Cip1}$-dependent pathway has been postulated that would still operate as a further (redundant) safeguard mechanism against oncogenic challenges which needs to be molecularly defined.

The p53-$p14^{ARF}$-HDM2 and Rb-$p16^{INK4a}$-cyclinD-CDK4 pathways

The great majority of malignant brain tumors harbor inactivating mutations in both the p53- and the Rb-related pathways, which exert cardinal cellular functions [33, 34, 40, 50, 51, 61]. Each of these functionally linked and physically interacting proteins can be targeted by the stochastic mutation selection process during tumorigenesis. Many functions have been assigned to the p53 protein. Simplistically, p53 is a short-lived transcription factor which is upregulated in response to cellular stresses such as UV and γ irradiation, double-strand breaks, and

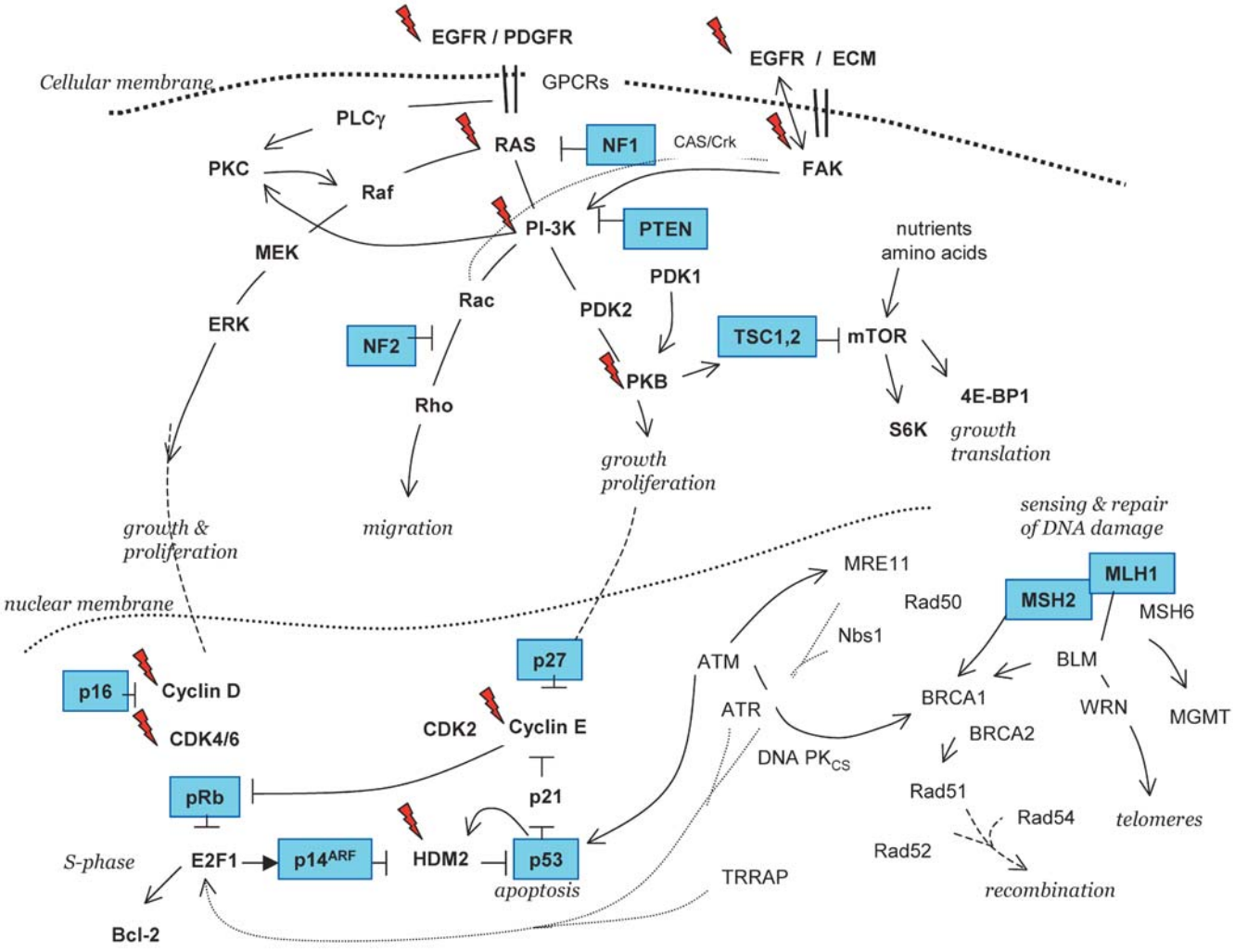

Fig. 3 Signaling network in gliomas. Upregulated growth factor receptors transmit migratory, growth, and survival signals via the Ras/MEK/ERK, PI-3 K/PKB, Rac/Rho, and mTOR/S6 K pathways into the nucleus, where a unique neoplastic transcription program is induced. Tumor cells have acquired the ability to evade cell cycle arrest and apoptosis. Moreover, sensing and repair of damaged DNA is impaired, leading to accumulation of genomic instability. The tumor suppressor genes that give rise to familial brain tumor syndromes and induce glial brain tumors are shown in blue boxes (NF-1, NF-2, PTEN, TSC1,2, p53). Frequently altered cell cycle and apoptosis regulators in sporadic glioblastomas are also marked in *blue boxes* (Rb, p16, p14, p27, HDM 2). The red arrows denote oncogenic gain of function alterations. The DNA damage sensing and repair pathway is outlined, also marking the mismatch repair proteins hMSH2 and hMLH1 that give rise to the rare Turcot's syndrome

cytotoxic drugs that either facilitates DNA repair by halting the cell cycle or, if the amount of damage is beyond the cell's capacity for repair, inducing cell death [11]. p53 can be viewed as an apoptostat, a protein that sets a cell's apoptotic threshold in response to specific endogenous and exogenous challenges. The Rb-E2F1 pathway controls the transition from the G1 into the S phase, and mutations that inactivate either Rb or $p16^{INK4a}$ or activate cyclin-dependant kinase 4 (CDK4) or cyclin D lead to deregulation of the transcription factor E2F1, which induces the expression of S-phase-related genes [40] but also leads directly to the expression of the antiapoptotic gene Bcl-2 [36]. The counterintuitive finding, however, that E2F-1 was underexpressed on the protein level in the majority of gliomas will have to be further analyzed [16]. $p16^{INK4a}$, which is elevated during cellular senescence, acquires immortalizing mutational inactivation during tumorigenesis to evade senescence-like growth arrest, which can be elicited in response to sustained activation of the Ras/Raf/MEK/ERK pathway [73]. Senescence-like growth arrest pathways could be induced in normal human astrocytes in response to the expression of Raf-1 in $p16^{INK4a}$-expressing and -deficient cells, as discussed above [28].

The p53 and Rb pathways have been extensively reviewed, including the associated familial cancer syndromes [34, 51, 93]. In this review, the dual role of the INK4a/ARF locus on human chromosome 9p21 shall briefly be highlighted. It represents a curious case of a single genetic locus giving rise to two alternatively

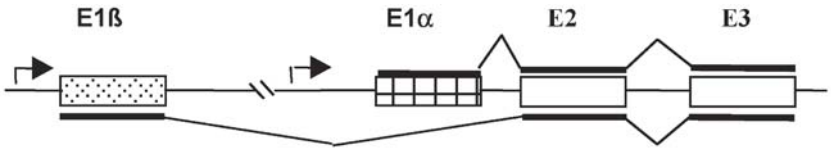

Fig. 4 Genetic structure of the INK4a/ARF locus on chromosome 9p21. The $p16^{INK4a}$ gene is composed of exons 1α, 2, and 3, while the human $p14^{ARF}$ (p19 in mice) uses a different promoter of exon 1β about 20 Kb upstream of exon 1α. The genomic sequences of both transcripts are entirely different, giving rise to two distinct small cell cycle regulatory proteins, because exon 1β uses an alternative reading frame

spliced transcripts with entirely different reading frames, $p14^{ARF}$ (in mice $p19^{ARF}$) and $p16^{INK4a}$ (Fig. 4) [105]. The human $p14^{ARF}$ gene was accidentally discovered during gene expression analysis of the methylated and unmethylated $p16^{INK4a}$ gene [79, 84] which was almost simultaneously followed by elucidation of the structure and function of the murine $p19^{ARF}$ protein [94]. Since $p16^{INK4a}$ negatively regulates CDK4 and $p14^{ARF}$ inhibits HDM2, blocking rapid ubiquitin-mediated decay of p53, simultaneous inactivation of both genes by a homozygous deletion deregulates both the Rb and p53 pathways. The biological function of this complex locus has been thoroughly analyzed in gene knockout experiments in mice, creating animals by either selectively inactivating $p19^{ARF}$ or $p16^{INK4a}$ or by simultaneously targeting both genes [55, 59, 101, 102]. These animal data reveal subtle differences, even arguing for ARF haploinsufficiency to cooperate with inactivated $p16^{INK4a}$ and giving rise to metastatic melanoma [102]. Is the INK4A/ARF locus a glioma inducer? Not really, because the $p19^{ARF}$-only mice displayed glioma-type lesions at low frequency [55]. However, in mice and humans alike, this locus efficiently drives tumor progression. According to a recent study using highly reliable quantitative real-time polymerase chain reaction (PCR) to circumvent the difficulty in assessing homozygous deletions in primary tumor tissue, some 50% of malignant human gliomas were found to delete the INK4A/ARF locus, displaying homozygous deletions that span the reading frame of both genes [61]. This alteration is therefore one of the most frequent genetic changes operative in glioblastomas that is acquired during tumor progression and might be linked to worse prognosis in patients older than 50 years of age [61]. Similar observations were made in a recent murine oligodendroglioma and mixed glioma model overexpressing PDGFR-B under both nestin and GFAP promoters. The Ink4a/ARF –/– background was not tumorigenic per se but impressively increased the malignancy from low-grade to anaplastic stages [21]. Most human malignant gliomas that retain the INK4a/ARF locus display mutations in other genes of the p53 and the Rb pathway which both are dysregulated in the great majority of tumors, leading to an unchecked cell cycle and resistance against apoptotic stimuli.

The role of phosphatase tensin homology and PKB/Akt-1

Germline mutations of the tumor suppressor gene phosphatase tensin homology (PTEN) on chromosome 10q23.3, initially also dubbed MMAC1 and TEP1, define a familial developmental and cancer syndrome whose diverse phenotypes have historically been ascribed to the Cowden-Bannayan syndrome and Lhermitte-Duclos disease [64, 65, 114]. The clinical spectrum comprises breast, follicular thyroid and prostate cancers, glioblastomas, meningiomas, skin and intestinal hamartomas, macrocephaly as well as enlarged cerebellar foliae, distorted cerebellar cortex, abnormally myelinated axon bundles, and dysplastic neurons [109]. A Dutch study that screened Cowden's disease patients for PTEN mutations suggests that the phenotype can be caused by at least one distinct additional gene in kindreds with wild-type PTEN sequence [89]. Several murine PTEN-deficient mouse models have been generated that in part reiterate the clinical phenotype seen in humans [5, 23, 60, 92, 115]. Interestingly, the neurological spectrum of these mice includes macrocephaly, seizures, and enlarged neuronal soma size without increased proliferation, while Akt-1 is hyperphosphorylated [5, 60]. PTEN contains a central catalytic phosphatase core domain that negatively regulates phosphoinositol-3 kinase (PI3K) by dephosphorylating phosphatidylinositol-3,4,5 triphosphate (PtdIns-3,4,5-P3) and phosphatidylinositol-3,4 diphosphate [75]. In contrast to an earlier report [117, 118], focal adhesion kinase (FAK) could not be confirmed as a substrate of PTEN using the same experimental conditions [78]. Furthermore, the dominant negative inhibitor of Tyr-397 FAK was not found to be PTEN-dependent [53, 54]. The N-terminal domain of PTEN is homologous to the cytoplasmic proteins tensin and auxilin, which interact with actin filaments at focal adhesions and clathrin-coated vesicles. In case of mutant PTEN, the elevated lipid second messenger PtdIns-3,4,5-P3 is used by PI3K to hyperphosphorylate protein kinase B (PKB)/Akt [76], which modulates the activity of proteins that play a critical role in cell survival, invasion, and proliferation [47, 78]. The catalytic activity toward phosphoinositide substrates is required for growth suppression, and PTEN-mediated growth inhibition is due to a G1 cell cycle block rather than to induction of apoptosis [35]. The PTEN structure shows a phosphatase domain similar to that of protein phosphatases but with an enlarged active site to accommodate the phosphoinositide substrate, and it also

reveals that PTEN has a C2 domain [63]. The PTEN C2 domain binds phospholipid membranes in vitro, and mutation of basic residues that could mediate this reduces PTEN's membrane affinity and ability to suppress the growth of glioblastoma tumor cells. PTEN is also a powerful inhibitor of cell motility which was found to be independent of its catalytic domain [78] and might be linked to the N-terminal tensin and auxilin homology domains. Mutant PTEN might be linked to an aggressive clinical course [68, 99]. On the other hand, human and murine tumors with an activated PI3K/PTEN/Akt pathway might be exquisitely sensitive to rapamycin, a macrolid antibiotic that downregulates mTOR and potentially opens a new therapeutic window for these tumors [22, 121]. Of note, the tumor suppressor genes TSC1 and TSC2 that give rise to hereditary tuberous sclerosis—mainly a hamartomatous developmental disorder with occasional manifestation of giant-cell astrocytomas—are negative regulators of the mTOR pathway (Fig. 3) [87].

Murine glioma models mimicking the human disease

Striking progress in cancer genetics and transgenic and knockout technologies has allowed the creation of animal models that faithfully reflect most phenotypic hallmarks of the human disease. Several models have recently been presented that highlight the critical role of the cancer genes defined in the human disease and draw our attention to the developmental origin of the different glial neoplasms and the effect of growth factor signaling. A classification scheme based on genetically engineered gliomas in mice has been recently presented that summarizes the histopathological findings in relation to genetic manipulations [72].

The oligodendroglioma and mixed-glioma mouse

In a cell culture and murine model, it was recently shown that PDGF autocrine stimulation dedifferentiated cultured astrocytes and induced oligodendrogliomas and oligoastrocytomas from neural progenitors and astrocytes [21]. These experiments were performed using Ink4a-Arf+/+ and Ink4a-Arf–/– mice, showing that deletion of this dual tumor suppressor locus was not required for PDGF-induced glioma formation. The INK4a-Arf-null background, however, led to a more malignant phenotype with areas of necrosis and neovascular proliferation in the PDGF-β-induced oligoastrocytomas and oligodendrogliomas. In an earlier report, brain tumors could be induced in mice using a similar rationale based on recombinant platelet-derived growth factor beta-chain retrovirus which stimulated PDGFR alpha expression within an autocrine loop [119, 120]. In this model, tumors were more malignant, displaying characteristics of glioblastoma multiforme or a primitive neuroectodermal tumor. The consistent expression of nestin suggested that they were all derived from an immature neuroglial progenitor. PDGFR might be linked to oligodendroglial differentiation, since in another model, transduction of Ink4a-ARF-deficient neural progenitor cells with mutant ΔEGFR induced glioblastomas but not oligodendrogliomas (personal communication, Monika Hegi).

The RAS-Akt glioma mouse

In this transgenic model, genes encoding activated forms of Ras and Akt were transferred to astrocytes and neural progenitors in mice in a tissue-specific manner [45]. Neither activated Ras nor Akt alone was found sufficient to induce gliomas; however, the combination of activated Ras and Akt induced high-grade gliomas with the typical histological features. These tumors manifested after transducing neural progenitor cells but not differentiated astrocytes.

The NF-1-p53 glioma mouse

Three critical factors constituted the basis for this model: firstly, NF-1 knockout mice display increased levels of gliosis; secondly, p53 is mutated in human astrocytomas; and thirdly, NF-1 patients develop astrocytomas. This model was generated by a gene knockout strategy [95]. Instead of transferring mutant Ras into a precursor cell, the NF-1 gene neurofibromin, which negatively regulates Ras as a Ras-GAP, was used to generate this mouse model by crossing NF-1-deficient mice with heterozygous p53 knockout mice. Both genes lie on the same chromosome only 7 cM apart, and a recombinant mouse line was selected which harbors both genes in the cis position. Thus, the second step of inactivating the wild-type allele by loss of heterozygosity (LOH) simultaneously inactivated both genes. In this model, all typical features of astrocytoma progression from WHO grades II–IV were observed, including invasion, new vessel formation, multinucleated giant cells, necrosis, and subpial, perivascular, and perineuronal satellitosis. As predicted for a tumor suppressor, the wild-type allele was found to be inactivated by LOH. This is a remarkable experiment, exploiting gene knockout strategies and cancer genetics in mice requiring no further manipulation. Penetration of the disease phenotype might be enhanced by breeding these mice with genetically unstable animals.

The Ras-only astrocytoma model displays genetic instability

In contrast to the mouse model presented by Holland et al. [45] in which an activated Ras gene alone was not tumorigenic but only in combination with activated Akt, a mouse model was recently introduced that gives rise to rapidly growing malignant astrocytomas relying only on activated Ras [24]. In this model, transgenic animals were created as chimeras taking advantage of transformed

embryonic stem cells that overexpress mutant Ras under a GFAP promoter. Two tumor-derived cell lines were shown to harbor alterations typically seen in human disease such as loss of $p16^{INK4a}$ and $p19^{ARF}$ expression and high levels of CDK4 and MDM 2 due to trisomy 10, which is syntenic to the human 12q13–14 region, and in one case loss of the PTEN protein. It has to be clarified whether the fundamental difference between these different animal models with regard to the oncogenic potential of the mutant Ras gene is simply due to variations in the genetic background or if oncogenic activation of p21-Ras can indeed lead to genomic instability.

The therapeutic value of murine glioma models

Taken together, these animal models represent very significant progress in mimicking distinct features of the human glioma phenotype by pinpointing the tumor-inducing factors and pathways which are preferentially targeted in human disease. It is an open question whether these genetic murine glioma models will prove useful and reliable for assessing therapeutic strategies to treat human tumors or whether orthotopic models using human glioblastoma cell lines will yield more reliable results. A striking difference in human gliomas appears to be the level of genomic instability as compared to the single- and double-step tumorigenesis in these animal models, although an exhaustive search for additional genetic alterations possibly accumulated during tumorigenesis has not been reported, except for some typical cancer gene loci which were altered in Ras-only glioma mouse [24]. Even though faithfully reiterating most phenotypic features of human disease, the recombinatory potential of human tumor cells is likely to be quantitatively and qualitatively different from murine counterparts. Human tumor cells harbor a potent mutation generator (see below) which has not been described in these animal models so far. Genetic versatility allows human tumor cells to escape from virtually every therapeutic challenge. This caveat might be overcome by crossing some of these different glioma mice with animals harboring hemizygous mutations in so-called caretaker genes, e.g., mlh1 and msh2, or in recQ homologues such as blm or wernerin that are associated with cancer [74, 104].

The many possible causes of the glioma mutator phenotype—a missing pathway?

In contrast to cancer cells, DNA replication is exceptionally accurate in normal cells [71]. Considering the rarity of mutations in normal cells and the large number of mutations observed in human cancers, the spontaneous rate in the former cannot account for the large rate of the latter. Carcinogenesis involves substantial errors in DNA replication, deficits in DNA repair, and alterations in chromosomal segregation, giving rise to a mutator phenotype, as already envisioned in 1991 [70]. Conceptually, errors in DNA synthesis due to misincorporation of nucleotides by DNA polymerases and inadequate repair of DNA damage caused by exogenous or endogenous reactive molecules can lead to this phenotype. The overall mutation rate in somatic human cells has been estimated at 1.4×10^{10} nucleotides/cell per division, which translates into one mutant gene for each cell during an idividual's life span, based on 70,000 genes within the human genome. This implies that a few of the 10^{14} cells per human body will contain as many as 12 mutations, according to a Poisson probability distribution [71]. The slope of the exponential increase of cancer as a function of age suggests that there are six to 12 cancer-causing events [96], which could explain the increasing rate of cancer with age. This rate, however, cannot explain the high number of genetic alterations in cancer. The hundreds, maybe thousands of clonal mutations detectable in cancer tissue must occur due to a mutator mechanism leading to chromosomal instability, hyper- and aneuploidy, translocations, amplifications, loss of heterozygosity, and microsatellite instability.

Four major overlapping pathways for the repair of DNA lesions in human cells might be involved in the generation of a mutator phenotype: nucleotide excision repair, base excision repair, mismatch repair, and the direct reversal of lesions by recombination [44, 71]. In addition, subtly altered DNA polymerases might be causative in generating a mutator phenotype in some instances, e.g., one of the most inaccurate DNA synthesizing enzymes, DNA polymerase β, confers genetic instability and showed enhanced activity in several cancers [8]. Furthermore, the mutant DNA helicases BLM and WRN can be linked to cancer syndromes and premature aging characterized by increased formation of sister chromatid exchange in Bloom syndrome [74] or large deletions in Werner's syndrome [104], both uncommon autosomal recessive disorders. Future population-based genetic studies will show whether subtle alterations in any of these genes are linked to an increased incidence of brain tumors and whether sporadic tumors contain somatic mutations in any of these enzymes. A fundamental question is whether the inability to respond to DNA damage is a very early step in tumorigenesis that is a prerequisite for the malignant transformation.

The most dangerous type of DNA damage is the double strand break which results from exogenous agents such as ionizing radiation and certain chemotherapeutic drugs, endogenously generated reactive oxygen species, and mechanical stress on the chromosomes [57]. A single DNA double strand break can be sufficient to kill a cell if it inactivates an essential gene or triggers apoptosis. In addition, erroneous rejoining of broken DNA may occur, leading to the loss or amplification of chromosomal material or, under certain circumstances, translocations in which segments of chromosomal arms are exchanged, giving rise to tumorigenesis if a tumor suppressor or an oncogene is involved. Two distinct and conserved mechanisms for double strand break repair have evolved—the relatively error-free homologous recombination and error-

prone nonhomologous end-joining. Mammalian Rad51 interacts with the tumor-suppressor and cell-cycle proteins p53, Brca1, and Brca2, and other proteins have been linked to homologous recombination in mammals such as the protein kinase ATM [56], which is deficient in cancer predisposition, radiosensitivity, and the neurodegenerative syndrome ataxia-telangiectasia (A-T). Of note, A-T carriers (ATM heterozygotes) constitute approximately 1% of the general population and are believed to carry a 3–5-fold increased risk of developing breast cancer. In addition, ATM-related proteins (ATR) play a role in genomic integrity [2]. Following DNA damage, E2F1 is stabilized by ATM/ATR-mediated phosphorylation of the N terminus [69]. Indeed, somatic mutations in ATR have been detected in sporadic stomach tumors with microsatellite instability [83]. In the Werner's [104] and Bloom [74] syndromes, deficient DNA helicases cannot sufficiently suppress illegitimate recombination, which they normally prevent during homologous recombination. In addition, these DNA helicases are unique in their ability to disrupt G-quadruplex DNA, non-Watson-Crick structures that can form within guanine-rich DNA sequences such as telomeric repeats, which is critical for telomere maintenance [66]. The mismatch repair protein MLH1 has recently been found to interact with BLM in a poorly defined postreplicative repair mechanism, since no microsatellite instability was detected in BLM-deficient cells [91]. Other proteins involved in double strand break repair are DNA protein kinases, phosphatidyl inositol 3-kinase-related kinases (PIKK), which includes ATM, ATR, and transactivation/transformation domain-associated protein (TRRAP9, and nibrin (Nbs1) and MRE11, genes mutated in the ATM-like disorders [15]. MRE11 associates with E2F family members via the Nbs1 N terminus, suppressing double strand breaks [20] by its influence on regulation and progression of DNA replication (Fig. 3) [80]. TRRAP is a cofactor of c-Myc- and E2F-mediated oncogenic transformation, part of histone acetyltransferase complexes, and essential for cell cycle progression [41, 82]. The large number of proteins that participate in the recombination machinery, for example altering the acetylation state of histones or remodeling chromatin, can be regarded as members of the class of "genome caretakers" which act to maintain genetic stability.

Our knowledge about the involvement of DNA repair genes in glioma tumorigenesis is limited. It has been shown that gliomas are surrounded by normal cells defective in the repair of DNA damage by alkylating agents, implying the lack of O_6-methylguanine-DNA methyltransferase (MGMT) production as a predisposing factor for the development of brain tumors [107]. In contrast, MGMT protein is significantly elevated in malignant brain tumor tissue, conferring resistance towards alkylating agents [108]. A correlation between MGMT expression and chemoresponse was also described in temodal-treated glioblastomas [31]. For homologous recombination-mediated repair of DNA damage induced by alkylating agents, mismatch repair cooperates with MGMT and might be epigenetically inactivated, as shown for hMSH6 [6, 123]. Apurinic/apyrimidinic endonuclease Ape-1 activity was described to be elevated, too, in human gliomas, also conferring resistance to ionizing radiation and alkylating agents [10]. Interestingly, the rare Turcot's syndrome, which is defined by comanifestation of colorectal cancer and glial neoplasm within the context of the hereditary nonpolyposis colorectal cancer syndrome (HNPCC), represents a true inherited mutator phenotype and can, in some glioblastoma cases [38], have a better clinical course [85]. Sporadic mismatch repair deficiency can rarely be detected in typical sporadic glioblastomas at a frequency clearly beyond 1% (unpublished observation). The mismatch repair phenotype is mostly caused by mutant human mutS homologue 2 (hMSH2) or mutH homologue 1 (hMLH1), which constitute the replicative multiprotein mismatch repair complex operative during cellular replication [52]. These two proteins have been found to be expressed at various levels in glioblastomas and not clearly correlated with therapeutic response to alkylating agents [31]. No clear correlation was detected for ATM expression and radioresistance in glioma cell lines, either [17]. An analysis of the genetic status of glioma patients was not performed; however, the role of ATM might be distinct in tumor induction at a very early stage as compared to radioresistance.

Practical consequences from understanding the glioma mutation generator

If indeed most "sporadic" gliomas occurred in a selected number of individuals that carry subtle mutations in enzymes responsible for the integrity of the genome, it would become possible to identify persons at risk by focused screening programs years before gliomas become clinically manifest. Such a scenario is realistic, even though tools for detecting early disease stages will have to be improved. Treatment aiming at eradicating full-blown glioblastoma appears highly difficult, given the infiltrative nature of the disease, which harbors invasive cell clusters deeply beyond visible tumor margins. Early detection might render glioblastomas amenable to more successful interventions at more favorable stages with much lower tumor burden.

Unchecked invasiveness—a key factor of glial malignancy

Growth factors and Rac-dependent migration

PDGF can stimulate migration of oligodendrocyte progenitor cells [110], neonatal rat cortical astrocytes [13], and embryonic rat neural stem cells [29]. PDGF signals through PI3K and PLC-γ [98]. EGFR autocrine signaling induces cell scattering and migration in glioma cells in vitro [53], and glioma cells expressing mutant ΔEGFR

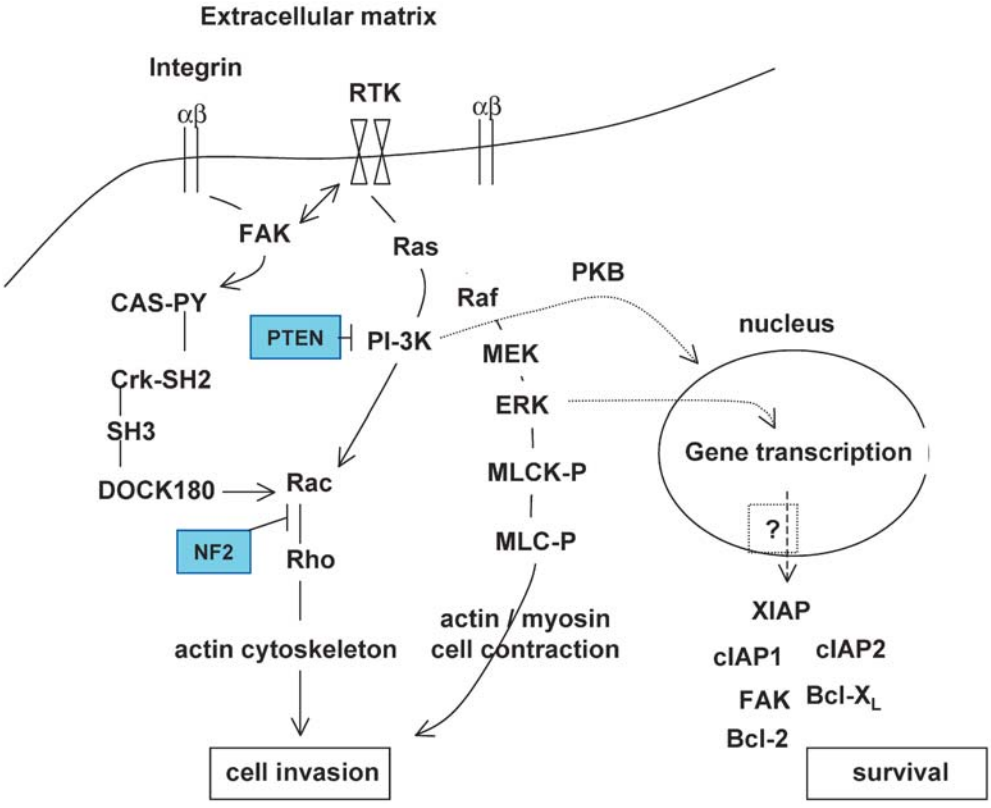

Fig. 5 Migration and survival pathways in gliomas. The major receptor tyrosine kinases EGFR and PDGFR mediate migratory and survival cues into the cell via FAK/CAS/Crk/Rac and Ras/MEK/ERK. ERK enters the nucleus and induces a promigratory program but also promotes invasion via MLCK. ERK and PKB change gene transcription, which is possibly responsible for altered expression of some inhibitors of apoptosis and Blc-homologous genes

show enhanced invasiveness when implanted in mouse brain [86]. G-protein-coupled receptors play a role in growth factor-mediated migration by cross-talking with the tyrosine kinase receptor in a positive feedback loop and enhancing signaling via the small GTPase Rac [43]. Of note, Rac seems to be negatively regulated by merlin, the neurofibromatosis type II (NF-2) tumor suppressor that predisposes humans and mice to tumor development [103]. The NF-2-encoded protein, merlin, belongs to the ERM (ezrin, radixin, and moesin) family of cytoskeleton:membrane linkers (Fig. 3, Fig. 5).

p16^{INK4a}, PTEN, and invasion

PTEN modulates migration via PIP3 levels as a negative regulator of PI3K that channels into the PI-3K-Rac-Rho pathway [19, 109]. In addition, PTEN has been shown to strongly suppress glioma invasiveness independently of its catalytic domain, a finding that requires further experimental workup [78]. Invasion also appears to be regulated by p16^{INK4a}. In a recent study, expression of full-length p16^{INK4a} was found to block $\alpha_v\beta_3$ integrin-dependent cell spreading on vitronectin, ascribing a novel function to the p16^{INK4a} tumor suppressor protein in regulating matrix-dependent cell migration [27].

Integrin and EGFR signaling converging at the focal adhesion kinase

Glioma cells perform their widespread and devastating invasion of normal brain tissue along white matter tracts and perivascular spaces. These tumor cells are resistant to anoikis, reflecting independence from integrin signaling. In normal cells, loss of substrate attachment leads to the induction of apoptosis, or anoikis [32]. There are obviously differences in invasiveness between different grades and from tumor to tumor which are difficult to assess objectively, as there are no parameters yet to measure the extent and dynamics of tumor cell infiltration. One such parameter is FAK, which appears to be a key mediator of cell shape and motility. It is required for integrin-dependent signaling and modulates cellular adhesion, migration [49], and survival [48]. With respect to tumor formation, decreased expression of FAK was shown to suppress papilloma formation during murine skin carcinogenesis [81]. FAK is targeted to the focal adhesions by the C-terminal focal adhesion targeting domain (FAT), promoting phosphorylation of tyrosine-397 (Tyr$_{397}$), which is essential for FAK activity. FAK could not be confirmed as a substrate of PTEN [78], as initially proposed [118]. In a recent analysis of the expression of FAK in human gliomas, FAK immunoreactivity was detected in all astrocytomas of grades II–IV and was significantly increased in glioblastomas and gemistocytic low-grade astrocytomas, which have a more ambiguous prognosis, while it was virtually absent from

the more "benign" oligodendrogliomas [54]. FAK was observed in all astrocytic cell compartments (cell soma and astrocytic processes) and often highly localized at the cell membrane. A low degree of FAK immunoreactivity in oligodendrogliomas is an intriguing observation that raises the question of whether FAK can be regarded as a useful prognostic marker to predict the dynamics of glioma invasiveness. Oligodendroglioma cells do also infiltrate the surrounding normal brain, however with different dynamics than those of astrocytic tumors. FAK expression appears to be related to tumor progression, since immunoreactivity was found to be higher in recurrent glioblastoma than in primary lesions. Increased FAK expression has been shown to increase expression of the inhibitor of apoptosis (IAP) gene family (cIAP1, cIAP2, XIAP) by activating PI-3 K/PKB and NF-κB, further elevating the apoptotic threshold [112]. Inactivating FAK using the C-terminal dominant negative FAT domain of FAK leads to decreased transcription of cIAP1, cIAP2, XIAP, and FAK itself in glioblastoma cells [54]. The expression of these inhibitory proteins of apoptosis is mediated by either PI3K/PKB and/or Ras/MEK/ERK (Fig. 5). The molecular mechanism of FAK upregulation in glioblastomas is not elucidated yet and could well result from a loss in function mutation of a regulatory gene, since no genetic alterations have been detected in the FAK gene (unpublished data, G. Reifenberger). Inactivation of FAK leads to attenuation of EGFR phosphorylation and enhances the degradation of EGFR in a ubiquitin-dependent way [54]. While some FAK effects are linked to EGFR signaling and could also be blocked by EGFR-specific tyrosine kinase inhibitors, the integrin-dependent part of the signal flow might be more directly blocked by targeting FAK. Two FAK-dependent pathways have recently been proposed that signal either via CAS/Crk/Rac to mediate invasion and survival or via Ras/Raf/MEK/ERK [18]. ERK further modulates myosin light chain kinase (MLCK) or directly enters the nucleus to alter gene transcription. A modified scheme is presented in Fig. 5. Fundamental cell fate decisions like migration and survival or anoikis are intimately linked and use several alternating pathways.

Conclusion

Dramatic progress in glioma genetics and tumor and developmental biology has allowed the establishment of several glioma mouse models that faithfully mimic hallmarks of the various glioma subtypes. Autocrine PDGF stimulation seems to favor oligodendrocytic differentiation, while Ras alone or in conjunction with an activated Akt allele gives rise to astrocytic tumors. Remarkable is a compound heterozygotic cis double knockout model in which both p53 and the negative Ras regulator neurofibromin are simultaneously inactivated, reiterating all stages of astrocytoma progression from WHO grades II to IV. The small inhibitory cell cycle regulators p27 and $p16^{INK4a}$/p14(p19 in mice)^{ARF} are not tumorigenic per se but act as powerful progressive factors accelerating proliferation and increasing malignancy in humans and mice alike [4]. The degree of genomic instability in these animal models might be quite different from the human disease and lead to distinct responses to pharmacological intervention. The genes that drive the glioma mutator phenotype and give rise to the increased mutational load await to be unraveled and might pave the way for early detection of these devastating lesions. Novel pharmacological approaches have started to exploit tumor-specific alterations of the cell signaling network, defining EGFR, PDGFR, FAK, PI-3 K, PKB/Akt-1, mTOR, and others as prime targets for small drug interventions.

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