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Identifying Candidate Genes Involved in Brain Tumor Formation

Review based on a doctoral thesis

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Abstract

Malignant primary brain tumors, gliomas, often overexpress both platelet-derived growth factor (PDGF) ligands and receptors providing an autocrine and/or paracrine boost to tumor growth. Glioblastoma multiforme (GBM) is the most frequent glioma. Its aggressive and infiltrative growth renders it extremely difficult to treat. Median survival after diagnosis is currently only 12–14 months. The present review describes the use of retroviral tagging to identify candidate cancer-causing genes that cooperate with PDGF in brain tumor formation.

Newborn mice injected intracerebrally with a Moloney murine leukemia retrovirus carrying the *sis*/PDGF-B oncogene and a replication competent helper virus developed brain tumors with many characteristics of human gliomas. Analysis of proviral integrations in the brain tumors identified almost 70 common insertion sites (CISs). These CISs were named brain tumor loci and harbored known but also putative novel cancer-causing genes.

Microarray analysis identified differentially expressed genes in the mouse brain tumors compared to normal brain. Known tumor genes and markers of immature cells were upregulated in the tumors. Tumors developed 13–42 weeks after injection and short latency tumors were further distinguished as fast growing and GBM-like. Long latency tumors resembled slow-growing oligodendrogliomas and contained significantly less integrations as compared to short latency tumors.

Several candidate genes tagged in this retroviral screen have known functions in neoplastic transformation and oncogenesis. Some candidates with a previously unknown function in tumorigenesis were found and their putative role in brain tumor formation will be discussed in this review. The results show that proviral tagging may be a useful tool in the search for candidate glioma genes.

Introduction

Brain tumor classification

Gliomas are primary brain tumors and are classified according to the World Health Organization (WHO) guidelines (*1, 2*). The incidence of malignant gliomas is about five to ten per 100000 people (*3*). The main types are astrocytomas, oligodendrogliomas, ependymomas and mixed oligoastrocytomas, usually characterized by different histopathology, a procedure first described in 1926, by Harvey Cushing.

Astrocytomas are divided into four grades after malignancy and account for 60 % of all primary brain tumors (*4*). Pilocytic astrocytomas belong to WHO grade

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I and are circumscribed slowly growing tumors that mainly occur in children or young adults. These tumors are often curable if resectable and different in genetic profile and clinical behavior compared to the more malignant diffusely infiltrating astrocytomas. Diffuse astrocytomas (AII, WHO grade II) are slowly growing tumors and have a high degree of cellular differentiation. Anaplastic astrocytomas (AA, WHO grade III) arise from lower-grade astrocytomas but have increased cellularity and mitotic activity. The most malignant, glioblastoma multiforme (GBM, WHO grade IV) is the most common of all brain tumors.

Characteristic histology of GBM includes nuclear atypia, mitoses, endothelial proliferation and/or necrosis (*4*). GBMs can be either primary, arising *de novo* without previous detection of a low-grade tumor, or secondary, after progression from a lower grade astrocytoma to a grade IV tumor. By comparison, primary glioblastomas are more frequent and affect older people than secondary GBMs. Brain tumors stay within the central nervous system (CNS) and very rarely metastasize, in contrast to other malignant solid tumors.

Oligodendrogliomas are of grade II or III and consist of well-differentiated diffusely infiltrating tumor cells morphologically resembling oligodendrocytes (*1*). Over the last ten years the incidence of oligodendrogliomas has been reported to increase from the classic figure of 5% up to 25% of all glial tumors. This change is suggested to be caused by mislabeling of some oligodendrogliomas or mixed oligoastrocytomas that previously were classified as astrocytomas (*5, 6*).

Oligoastrocytomas are clonal tumors, grossly subdivided from their pathological and molecular relation to astrocytomas or oligodendrogliomas.

Although they are rare compared to neuroepithelial tumors overall, ependymomas represent the third most common brain tumor in children. Most pediatric examples arise intracranially whereas ependymomas found in adults are evenly distributed between the brain and spinal cord.

Embryonal CNS tumors like the medulloblastoma (MB) and the supratentorial primitive neuroectodermal (sPNET) brain tumor are the most common malignant pediatric brain tumors. Medulloblastomas are invasive cerebellar neoplasms. The sPNETs are entirely supratentorial and often located in cerebrum.

Molecular biology of brain tumors

As has been emphasized in recent reviews (*5, 7–10*), increased tyrosine kinase signaling and loss of cell cycle control are important hallmarks of gliomas as well as cancer in general (*11*). Genetic analyses have delineated differences in molecular alterations between primary and secondary glioblastomas and oligodendrogliomas (Figure 1).

Growth factor pathways

Platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) are ligands for receptor tyrosine kinases with essential roles in brain tumor development. Other growth factors involved in brain neoplasms are insulin-like growth factors,

IGFs (*12*), fibroblast growth factor 2, FGF2 (*13*), ciliary neurotrophic factor, CNTF (*14*), hepatocyte growth factor/scatter factor, HGF/SF (*15*), vascular endothelial growth factor, VEGF (*16*) and transforming growth factor-β, TGF-β (*17, 18*).

EGF and other ligands, like transforming growth factor-alpha (TGF-α) activate members of the epidermal growth factor receptor family (ErbB/HER1-4). The most studied is the epidermal growth factor receptor (EGFR, HER1 or c-erbB1) that was early recognized in gliomas (*19, 20*). Approximately half of GBMs overexpress EGFR. About 40% of those tumors express activating deletions of EGFR; most often seen is the EGFRvIII type generating a receptor that signals independent of ligand binding (*21, 22*). As suggested in Figure 1, increased EGFR signaling is more common in primary GBM (*23*). Although receptors for PDGF and EGF activate similar signaling pathways (see Figure 3) they can actually be coexpressed in glioma cells (24).

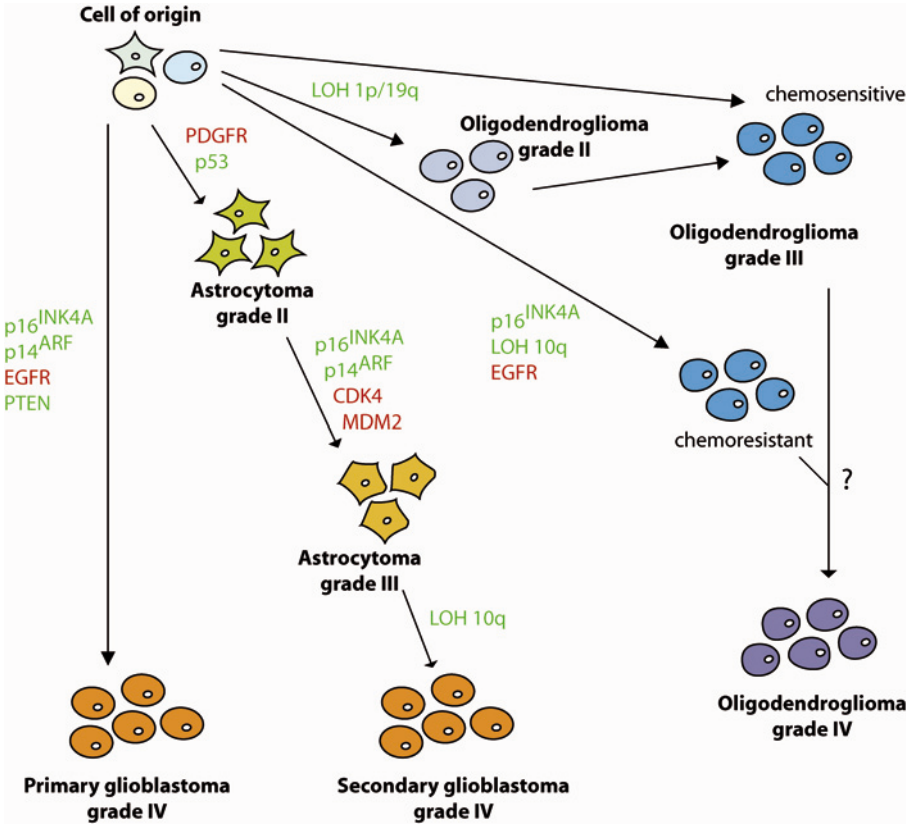

Figure 1. Suggested molecular pathways leading to human glioma formation and progression. The genetic alterations are a subset of those found in these tumors that correlate with grade and type of tumor. They are presented as activated (red) or inactivated (green) along arrows. LOH: Loss of heterozygosity. Adapted from (5).

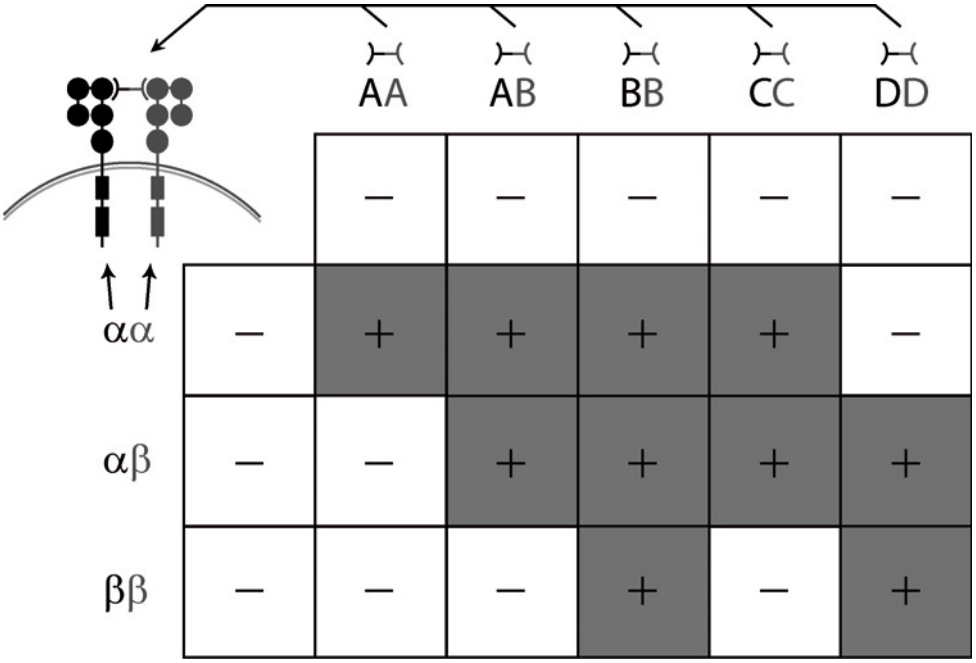

Figure 2. The three known PDGF receptor isoforms (α:α, α:β, β:β) have different binding capacities for the five known dimeric combinations of the PDGF ligands A-D. The possible pair wise receptor:ligand combinations are shown (boxes). Only the combinations shown in grey with a plus sign will activate an autocrine stimulatory loop.

PDGF-induced transformation

PDGF is a mitogen of many cell types of mesenchymal origin, like cultured fibroblasts and smooth muscle cells. PDGF is also a mitogen of cell types of the neuroectodermal origin like glial cells (25–27) and neural stem cells of embryonic (28) and adult (29) origin. The family of platelet-derived growth factors consists of four members, PDGF-A, -B, -C and -D. Through biosynthesis and processing they can form five dimeric isoforms named PDGF-AA, BB, AB, CC and DD (30, 31). For these disulphide-linked ligands there are two receptor tyrosine kinases, the PDGF α-receptor and the PDGF β-receptor that can form three different dimers (α:α, α:β, β:β).

In a matrix of ligand:receptor combinations, ten combinations can form an autocrine stimulatory loop necessary for function (Figure 2). Overexpression of PDGF ligands and receptors has been suggested to be one of the earliest alterations in secondary GBM development and found in low-grade astrocytomas and consistently in gliomas of higher grade (32–36). Analysis of expression of PDGF ligands and receptors in human gliomas suggests that there is an autocrine stimulatory loop in almost all gliomas (37).

The potential of PDGF as an initiator of brain tumors came from the studies of the simian sarcoma virus (SSV). The transforming protein of SSV is essentially

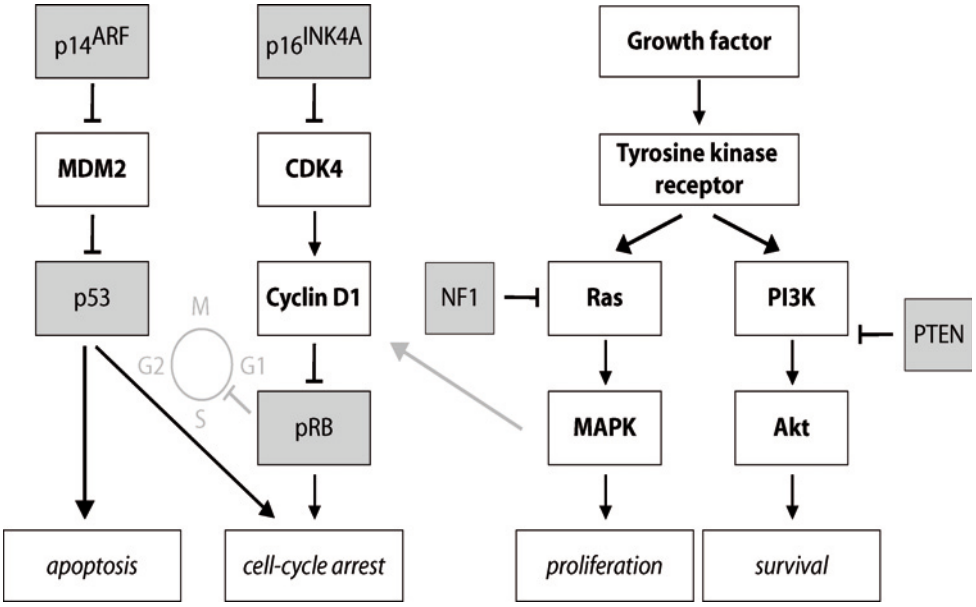

Figure 3. Major molecular pathways altered in gliomas. Several pathway elements and signaling interactions have been removed leaving a greatly simplified linear presentation. Elements with bold letters are activated through overexpression, amplification or activating mutation. Pathway elements in grey are frequently inactivated through deletion, mutation or promoter methylation. Adapted from (9).

identical to the PDGF B-chain (38, 39) and the virus was found to induce glioblastomas when intracerebrally injected in marmosets (40).

Cell cycle regulators

The eukaryotic cell cycle is divided into four phases. G_1 is the initial gap phase where the cells prepare for DNA synthesis occurring in the S phase. These steps are followed by the coordinating gap phase G_2 and chromosome separation and cell division (mitosis) in the M phase. Transformed cells are damaged in their ability to control entry into S-phase allowing for uncontrolled cell growth.

The cyclin dependent kinase (CDK)-cyclin D/INK4/retinoblastoma (pRB)/E2F pathway controls the transition from G_1 into S and is altered in about 80% of all human neoplasms (41). In GBMs, most of the mutations are seen as deletions in the INK4A gene, *CDKN2A* (42), which encodes the proteins $p16^{INK4A}$ and $p14^{ARF}$ (Figure 1 and 2). Mutations that inactivate either pRB or $p16^{INK4A}$ or activate cyclic-dependent kinase 4 (CDK4) or cyclin D induces the expression of S-phase related genes by deregulating the transcription factor E2F1. Mitogenic signals activate cyclin D-dependent kinases, which phosphorylate pRB and the pRB family proteins (p107 and p130). This facilitates entry into S-phase. Overexpression of CDK4 is seen in 15% of GBMs (43). In 40% of primary GBM, *INK4A/ARF* and *INK4B* on chromosome 9 are deleted (42).

Forced expression of $p14^{ARF}$, arrest cells in G_1 or induce apoptosis via *TP53* encoding the p53 protein (44). *TP53* is silenced by loss-of-function mutations in 40% of GBMs (45). These events (including allelic loss of chromosome 17p that harbors *TP53*) are equally observed in low-grade gliomas, anaplastic astrocytomas and secondary glioblastomas indicating that this is an early event in the development of these tumors. Inhibition of p53, that promotes genomic instability, is also regulated and processed by the MDM2/HDM2 (murine/human double-minute 2) protein that binds p53 and trigger its degradation by ubiquitylation. Tumors that do not have p53 depletion almost always show inactivation of $p16^{INK4A}$ or $p14^{ARF}$ instead (*7*).

Other aberrant signaling in brain tumors

Ras/MAPK (mitogen-activated protein kinase) and PI3K (phosphatidylinositol-3-kinase)/Akt signaling pathways by tyrosine receptor kinases lead to increased growth, metabolism, proliferation and survival (Figure 2). Unlike many carcinomas, malignant gliomas do not express mutant Ras (46). The suppressor gene, *NF1* (neurofibromin 1) is lost in neurofibromatosis type I, a common genetic disease of variable penetrance, which is characterized by nervous-system abnormalities including increased risk of benign and malignant brain tumors. NF1 acts to negatively regulate Ras by obstructing the activation of Sos (47, 48).

PI3K phosphorylation leads to the formation of PIP_3 (phosphatidylinositol-(3,4,5)-trisphosphate), which recruits proteins with pleckstrin homology domains (like Akt) to the cell membrane, where they can be activated. Activating mutations in the PI3Kα gene, *PIK3CA*, occurs frequently in various tumors like gliomas (49, 50). The proto-oncogene Akt is modulated by tumor suppressor protein PTEN (phosphatase and tensin homolog deleted on chromosome 10), a negative regulator of PIP_3 (51, 52) and found to be mutated in more than 30% of primary gliomas (53). Other pathways of cell scatter, migration and cytokine stimulation like PLC-γ (phospholipase C-gamma) and JAK-STAT (Janus activating kinase-signal transducers and activators of transcription) forwards cell signals downstream of RTKs (54).

The genes implicated in the genesis of medulloblastomas differ from those of gliomas. The two most studied pathways in MBs are sonic hedgehog (SHH)-patched (PTCH) and WNT signaling pathways although these pathways can be altered in gliomas as well (55). Individuals with *PTCH* mutations which activate the SHH pathway are more prone to get medulloblastomas (56). There is a high incidence of colon cancer but also MBs in Turcot's syndrome (57). This disease results from germline mutations in adenomatous polyposis coli gene that encodes a protein that regulates WNT. Both expression and tissue microarray studies of human medulloblastoma show that most of the patient samples express N-Myc (58). In addition, N-Myc has shown to be an essential target of SHH signaling (59, 60).

In most human brain tumor cells, unlimited cell division by telomere lengthening can be maintained through expression of the catalytic subunit of telomerase. Many gliomas that do not reactivate telomerase yet maintain telomere length suggesting an importance of the alternative lengthening of the telomeres pathway (61).

Solid tumor growth is dependent upon angiogenesis and one of the hallmarks

of high-grade gliomas is indeed microvascular proliferation. Necrosis and vascular hyperplasia are often seen in the rapidly growing GBMs. Cells in local ischemic and hypoxic environments activates the hypoxia inducible factor 1 (HIF-1) which activates VEGF receptors and other factors promoting angiogenesis (62). Finally, the machinery of DNA damage sensing and repair are most likely targeted during transformation.

Epigenetics and microRNAs

Mutation or deletion is not the only way by which a suppressor gene becomes inactivated. Modification of the genomic DNA by covalent attachment of methyl groups to cytosine bases (CpG islands) may efficiently silence tumor suppressor genes (63). These methylations (that do not alter the DNA sequence) often affect the promoters of the suppressor genes and are epigenetic mechanisms to control gene expression. These methylations can even be maintained during DNA replication, assisted by maintenance methylases, the enzymes that attach methyl groups to cytosine bases (64). The INK4A/ARF suppressor gene locus discussed above is deleted in about 40% of GBM samples. However, additionally 20% of the GBM tumors are found with a methylation silencing of the 5' CpG island of this gene (65). Another example is inactivation by methylation silencing of the O6-methylguanine-DNA-methyltransferase (AGAT) gene, MGMT which is associated with good outcomes in the treatment of glioma patients (see next chapter). Although these examples above concerns regions of hypermethylation it is rather hypomethylation that is the common feature of malignant cells. For example, there is a global decrease in 5-methylcytosines in human brain tumors relative to normal brain (66). A decreased methylation might activate normally silent oncogenes like e.g. PDGF-B. A recent report shows that TGF-beta promotes proliferation through the induction of PDGF-B in gliomas that have an unmethylated PDGF-B gene (67). In addition, hypomethylation might also lead to chromosome destabilization (68).

A microRNA is a small noncoding RNA molecule that targets the mRNA of protein-coding genes. The first microRNAs were discovered more than a decade ago (69). MicroRNAs are evolutionally reserved from plants to humans (70) implying that these microRNAs direct essential cellular processes. About 250 microRNAs have been reported in the human genome (71) although this is suggested to be a low estimation (72). These 18 to 25 nucleotide large microRNAs bind to complementary or partially complementary sequences of mRNA targets leading to a cleavage or a repression of the gene products. In this way microRNAs can control the expression of oncogenes or suppressor genes in the genome (reviewed in (73, 74).

Human microRNAs are frequently situated at genomic regions involved in cancer (75). Moreover, expression of microRNAs has in an impressive way been used to predict the survival outcome of cancer patients (76). The first report of a function of a microRNA in glioblastoma patients describes an aberrantly expressed microRNA-21 in patient samples that when inhibited is leading to apoptotic cell death of treated tumor cells (77). Continued research will probably reveal important mechanisms in tumorigenesis that is controlled by these small RNA molecules.

Brain tumor therapy

Glioma prognosis

Median survival of anaplastic astrocytomas and GBMs remains poor (2–3 years and 12–14 months respectively). Patients with oligodendrogliomas generally show a better prognosis and oligodendrogliomas with combination of LOH (Loss of heterozygosity) on chromosome arms 1p and 19q often respond better to chemotherapy (78) even after recurrence (79). In GBM, TP53 mutations and LOH on 10q emerge as favorable and poor prognostic factors, respectively. GBMs with the combination of LOH on 1p and LOH on 19q are less malignant and could not be morphologically distinguished from other GBMs (80).

Molecular profiling has been shown to correlate better with survival than histological diagnosis (81). A recent report classifies high grade gliomas into distinct subtypes from their molecular signature and prognosis (82). They further suggest that Akt and Notch signaling are hallmarks of survival in high grade gliomas and that the aggressiveness of these brain tumors are regulated by similar processes that control forebrain neurogenesis. These reports are convincing and suggest molecular profiles instead of histopathology to better assign gliomas to particular categories and therapeutic groups. The problem today is that clinical departments neither have the equipment nor the personnel needed to test for such tumor markers routinely; at least not yet.

Glioma treatment

In standard treatment protocols, brain tumor resection and radiation therapy are followed by chemotherapy with drugs causing DNA alkylation, like nitrosoureas. Standard treatment is a combination of procarbazine, lomustine and vincristine or carmustine or temozolomide alone (83). Until recently, the benefit of chemotherapy following surgery and radiation has been negligible for most GBM patients. However a study indicated that GBM patients with a methylated MGMT promoter, which is about 45% of GBM cases, benefit from temozolomide treatment (84, 85). This case was recently followed up by a randomized, Phase III study including 573 GBM patients. Patients treated with temozolomide after radiation had a median survival of 14.6 months as compared to 12.1 months for patients given radiotherapy alone (86). These results made this treatment scheme to become the standard of care for patients with GBM and approved in USA and Europe for newly diagnosed GBM.

Almost all treated high grade astrocytoma cases recur and the tumor usually arises within 2 cm of the prior resection margin (87). The current treatment strategies for recurrent astrocytoma have recently been reviewed (88). Available therapies following progression are considered ineffective with a progression free survival after six months (PFS-6) of less than 15%. That is now the commonly used end point to assess therapeutic activity in clinical oncology of recurrent glioblastomas (89).

Eventually, effective supportive care of GBM patients is important. Symptomatic treatments e.g. cerebral edema treatment with glucocorticosteroids as well as rehabilitation and psychological support are necessary.

New drugs and future therapy

Several new drugs to treat gliomas are currently in clinical trials. The focus is mainly on molecular targets like tyrosine kinase receptors and downstream effectors (90, 91). For example, receptor tyrosine kinase inhibitors, like imatinib mesylate (Gleevec®) inhibiting PDGF receptors are tested in several clinical studies for glioma treatment. Phase II studies with imatinib in monotherapy in recurrent gliomas have shown minimal activity (92). However, combination therapy with hydroxyurea and imatinib resulted in a 24–32% PFS-6 (93–95). The mechanism of this co-operating antitumor activity in some of these patients is unknown. However, although drugs are able to penetrate the blood-brain barrier, they may not be able to target all tumor cells due to the high interstitial fluid pressure in the tumor. Imatinib has been reported to decrease this pressure (96) which can enable a more efficient drug delivery.

Gefitinib (Iressa®) and erlotinib (Traceva®) are selective EGFR inhibitors enrolled in several clinical studies. Only seven (13%) of 53 recurrent GBM patients had PFS-6 from gefitinib treatment (97) and the drug have shown little effect on cells expressing the mutated EGFRvIII. However, erlotinib treatment has shown to have promising effects in GBMs where EGFRvIII and PTEN are coexpressed (98).

Bevacizumab is a humanized monoclonal antibody to VEGF that is approved for colon cancer in the USA. Bevaciumab inhibits blood vessel formation and has in combination with irinotecan shown promising activity; 38% PFS-6 in recurrent grade III/IV gliomas (99) and as much as 46% PFS-6 in recurrent GBM patients (100). A strong antiedema effect and favorable PFS-6 was also found for the VEGFR inhibitor AZD2171 (cediranib) in recurrent GBM (101) which suggest that antiangiogenic agents are indeed likely to play a role in high grade glioma management.

Further, many promising inhibitors are targeting mTOR, acting downstream of PI3/Akt signaling. However, clinical trials suggest that single agent mTOR inhibitors have minimal activity in GBM (102).

No subset of human malignant gliomas has been shown to depend on a single oncogene or tumor suppressor. This might reflect the limitations in clinical trials that target only one oncogenic pathway. As many receptor tyrosine kinases are known to be coactivated in glioma cells (24) second generation clinical trials have to include two or three combinations of inhibitors of these critical signaling pathways in glioma. Another approach is to continue combining different molecular-targeted agents with standard cytotoxic agents (*103*).

Several molecules have been successful in preclinical evaluation of brain tumors. The targeting of Shh pathway in medulloblastoma with small molecular inhibitors dramatically improved the survival of tumor-bearing *Ptch* mutant mice (*104*). This is only one example of a promising approach in future MB treatment.

Gene therapy, immunotherapy and e.g. oncolytic viruses that can specifically replicate and lyse in tumor cells (*105*) can be used in glioma treatment. A similar strategy of gene therapy using retrovirally transduced lymphocytes to treat patients with metastatic melanoma (*106*) could for example be worth trying.

Strong efforts are made to effectively target tumor stem cells that will be dis-

cussed in the next chapter. These cells are believed to survive both radiation and chemotherapy and can generate new tumor cells during recurrence.

Development of brain tumors

The classic theories of gliomagenesis are based on histological classification, morphological appearance and protein expression patterns of the bulk of the tumor cells. The focus has now changed as recent evidence suggests that gliomas may arise from a rare cell type with neural stem cell-like properties. These cells are those believed to persist after standard glioma therapy which makes them the ultimate targets for novel treatment strategies.

Cells of the brain

The brain is formed primarily during embryogenesis and until shortly after birth. The immature cells of the CNS, neuroepithelial stem cells, differentiate into different progenitor cells, which subsequently give rise to the three major cell types of the brain: astrocytes, oligodendrocytes and neurons (Figure 4). Of the three layers of the embryo: endoderm, mesoderm and ectoderm, the latter forms the nervous system and the skin.

In mouse CNS, by embryonic day (E) 8, driven by fibroblast growth factor (FGF), the multipotent stem cells of the ventricular zone form radial glial cells (in the intermediate zone) that guide neuronal and glial progenitors as they migrate away into the developing cortex (*107*). By E13, the stem cells become progressively more responsive to EGF, self-renew, and give rise to glial restricted progenitors (GRPs). By E18, the stem cells are responsive only to EGF and produce PDGFR-positive and PDGFR-negative glial restricted progenitors (*10*).

As neuronal generation and migration are complete, radial glial cells disappear or transform into astrocytes. This was thought to be an end process, until it was shown that differentiated astrocytes implanted in embryonic brain could dedifferentiate to radial glial cells (*113*). Correspondingly, embryonic cortical tissue implanted in an adult host produced new radial glial cells generated from the differentiated glial cells of the host adult brain (*114*). Suggested proteins required for this transformation are neuregulin 1 and erbB2 in concert with Notch signaling (*115*). Although a controversial issue, astrocytes are currently defined as stellate cells that contain the glial acidic fibrillary protein, GFAP (*116*). In addition, astrocytes are also derived at later stages from migratory progenitors from the dorsolateral subventricular zone (SVZ) (*117*).

The adult subventricular zone (*118*), the dentate gyrus (*119*) and the subcortical white matter (*120*) harbor residing neural stem cells. The SVZ lines the lateral ventricles of the forebrain and contain three main cell types: multipotent B astrocytes, A neuroblasts and type C precursor cells. The B cells are GFAP positive, *bona fide* SVZ stem cells that give rise to fast-cycling proliferating C cells that in turn

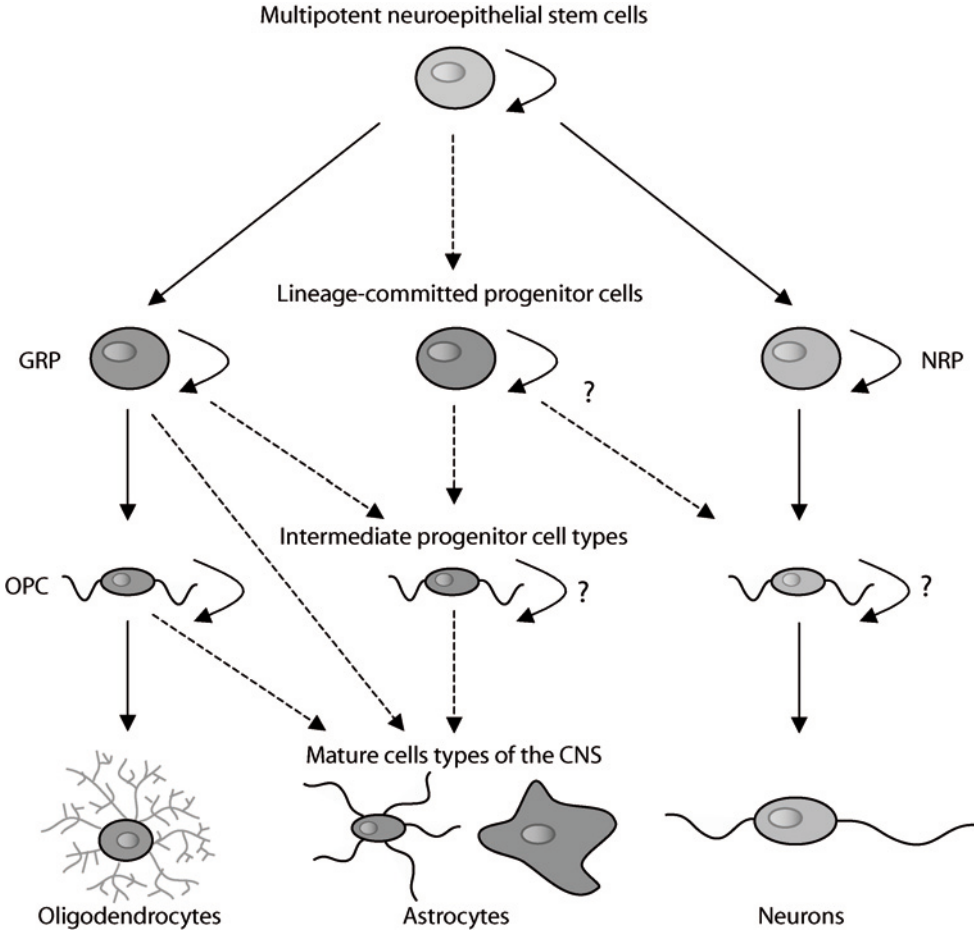

Figure 4. Linear relationship between cell types that could be the cells of origin for gliomas. Simplified overview that illustrates the increasingly recognized complexity of cellular differentiation during CNS development. Neuroepithelial stem cells generate linage-committed progenitor cells. Glial-restricted precursors (GPRs) are believed to generate oligodendrocytes and astrocytes whereas neuron-restricted precursors (NRPs) differentiate into neurons of different kinds (108). However, another line of investigation suggests that oligodendrocytes are more closely related to neurons (than they are to astrocytes) and there is an ongoing discussion of the existence of an oligodendrocyte-neuron precursor cell not represented in this scheme (109-111). Identification of other linage-restricted precursor cells, like astrocyte-restricted precursor cells, is of considerable interest. Curved arrows indicate self-renewal and question marks and broken lines indicate relationships that have yet to be confirmed. OPC: oligodendrocyte progenitor cell. Adapted from (112).

generates migrating A neuroblasts. The latter migrate away and settle down as new neurons in the olfactory bulb (*118, 121*).

In cerebellum, sonic hedgehog (Shh) protein regulates the self-renewal and the differentiation of the precursors of cerebellar granule cells. Similarly, Shh is both necessary and sufficient for oligodendrocyte induction in the spinal cord. Most oligodendrocytes, the myelinating cells of the brain, derive from restricted periventricular germinal regions of the neural tube (*122*). By E12.5 the first oligodendro-

cyte progenitor cells (OPCs) defined by expression of PDGFRα, Olig2 and Sox10, start to migrate away from the ventricular zone to colonize the gray and white matter. In addition, in the adult SVZ a recent report shows that B cells not only generate neurons but also new OPCs that give rise to oligodendrocytes (*123*). NG2 (chondriotin sulphate proteoglycan 4, neuron-glial 2) has been a reliable marker for OPCs, particularly when used in combination with PDGFRα (*124, 125*). About 8–9% of the adult brain consists of NG2-positive oligodendrocyte progenitors.

Glioma cell origin

When neural stem cells, with the capacity of self-renewal and differentiation into mature astrocytes and neurons, were found in the adult brain, an unexpected plasticity of the adult rodent and human brain was revealed (*119, 126*). The general view is that the origin of glioma is an early glial progenitor. These cells are predominantly localized in the subventricular zone or the dentate gyrus of the hippocampus. These regions seldom harbor any brain tumors in patients. However, the great proliferative and migratory capabilities of theses stem cells predict that tumor formation could occur anywhere.

GBMs are often heterogeneous, having both immature and differentiated areas. Among brain tumors, mixed oligoastrocytomas consist of areas containing cells that either resemble oligodendrocytes or astrocytes. Reports have observed loss of heterozygosity (LOH) of 1p and 19q in both of these areas, suggesting that both types of cells derived from a single precursor (*127*).

Several groups have identified stem-like cells in cultures of brain tumor patients (*128–130*). Interestingly, Singh et al. describe of an isolation of tumor cells expressing a CD133 cell surface marker (*131*). As few as 100 of these cells can, if serially transplanted into a nude mouse brain, reinitiate tumor growth and provide an exact recapitulation of the tumor from the patient. CD133-negative cells on the other hand can not induce brain tumors despite using as many as 10^5 cells. Remarkably, the method to sort out CD133 positive cells and use them as initiating tumor cells could be applied for medulloblastomas, glioblastomas and also ependymomas (*132*).

There is also a recent report where stem-like cells (B cells) express both PDGFRα and GFAP and are found to induce glioma-like lesions in adult mice treated with PDGF that might support this theory (29).

Replicating early glial cells, especially oligodendrocyte progenitor cells, are present in adult brain (*133–135*). These cells express PDGFRα (*136*) and are found in great numbers in the developing CNS. They have the ability to differentiate into oligodendrocytes (*137, 138*) but can be kept in a proliferating step *in vitro* by addition of PDGF together with FGF2 (*139*). A recent report describe that these NG2 cells generate tumors in adult rat white cortical matter upon retroviral infection with a shorter form of the PDGF-B ligand (*140*). However, the infected cells do not constitute all cells in the tumors as they recruit other progenitors in a paracrine fashion. The expression of PDGFRα as well as NG2 is often found in brain tumors (*141*).

Both transformed neural stem cells and OPC cells would indeed be able to cre-

ate all kinds of glioma types, including astrocytomas, oligodendrogliomas, mixed oligoastrocytomas as well as secondary glioblastomas (142). It is from these cell types crucial to sort out genes that are required for tumor formation. For example, a recent report stresses the importance of the Olig2 transcription factor and its requirement for glioma formation of *Ink4a/Arf*$^{-/-}$EGFRvIII neurospheres (143). When using the similar EGFR-driven orthotopic transplantation model (144), the Polycomb group gene and epigenetic silencer Bmi1 was reported to delay glioma development *in vivo* (145) when knocked out in Ink4a/Arf-deficient cells. Other examples include bone morphogenetic proteins like BMP4, that trigged a significant reduction in the neural stem-like, tumour-initiating precursors and abolished the capacity of transplanted human GBM cells to establish intracerebral GBMs in mice (146).

The origin of most medulloblastomas is thought to be the external granular layer (as discussed in (147)). However, recent evidence suggests that desmoplastic and classic MBs may have different origin (148). The desmoplastic but not the classic type is expressing similar markers as granule neurons. Hence, classic MBs are suggested to originate from the ventricular zone of developing cerebellum; the zone which contains multipotent stem cells (149).

Several reports have presented the astrocyte as a more plastic cell than previously thought, in fact suggesting it as a neural stem cell in disguise (150). The cell of origin of brain tumors is not known and one might envisage how appropriate mutations can lead to dedifferentiation of a mature astrocyte into a more immature state and generate a proliferating and migrating glioma clone. This theory is e.g. supported by a model where differentiated astrocytes were converted to a more undifferentiated state through retroviral transfection of EGFR activation and Ink4a/Arf loss in order to develop gliomas in mice (144). Committed glial progenitors can reacquire stem cell-like properties under certain conditions (151). In addition, a recent report presents that pluripotent stem cells can be generated from a few genetic changes in differentiated fibroblasts (152). Several important genes involved in cancer stem cell maintenance and "stemness" have been identified in the tumors of the nervous system (reviewed in (153)).

Viral tumorigenesis

Studies of the RNA and DNA viruses that cause tumors in animals and humans have contributed immensely to the understanding of cancer biology. Transforming retroviruses carry oncogenes derived from cellular genes. DNA tumor viruses encode oncogenes of viral origin that are essential for viral replication and cell transformation. SV40 (simian virus 40) is probably the most studied member of DNA virus belonging to the family of polyomaviruses. These viruses express T antigens that have been useful in identifying targets of cellular transformation in humans. The discovery of viral oncogenes (v-*onc* genes) and the realization that they were derived from cellular genes called proto-oncogenes (c-*onc* genes) provided strong

clues to explain the role of c-*onc* genes in other types of tumors. Many genes, including *Ras*, *Abl*, *ErbB*, and *Myc*, all first identified as v-*onc* genes, are now known to be activated in certain types of spontaneous tumors (154).

Retroviruses

The first reports of oncoretroviruses came in 1908 from Ellerman and Bang regarding avian leukosis and a few years later from Rous from studies of poultry sarcomas. Both groups showed that cell-free-filtrates from neoplasms of sick animals could induce new tumors when transferred to healthy animals. The family of retroviruses is subdivided into seven genera (155). The genome of simple retroviruses consists of three regions, *gag* (group specific antigen), *pol* (polymerase) and *env* (envelope). All retroviruses have the unusual reverse transcriptase (RT) reversely transcribing viral RNA into DNA (first described in 1970 (156, 157)) but also an integrase, necessary for integrating the virus into the host genome. When integration is completed, in the "proviral" state, the viral genes are flanked by two identical LTRs (long terminal repeats). Then the virus uses the host cell machinery to generate new viruses.

Regarding pathogenesis, oncoretrovirus could be subdivided in two groups; acutely transforming retroviruses, carrying oncogenes that induce fatal disease within approximately 2–3 weeks of infection and slow transforming retroviruses, generating tumors after a latency period of 3–9 months (158). Together with mouse mammary tumor virus (MMTV) inducing mammary carcinomas in mice, avian leukosis virus (ALV) and murine leukemia virus (MLV) are the most studied slow transforming viruses inducing lymphomas and erytholeukemias in birds and rodents, respectively.

With respect to their tropism, infections of retroviruses are limited to those cells that express the receptors recognized by the viral envelope protein. For example, ecotropic murine leukemia viruses infect murine cells, but not cells from other species whereas xenotropic viruses can only propagate in non-murine cells. Amphotropic or polytropic viruses are capable of infecting human, mouse or other cells (159).

The entry of ecotropic murine leukemia virus into cells requires the interaction of the envelope protein (Env) with its receptor, mouse cationic amino acid transporter 1 (mATRC1) (160). The corresponding gene encoding the receptor for ALV is *tv-a*, tumor virus A (161). In mouse cells infected with MLV, integration of viral DNA and production of viral proteins occur only after the cells traverse mitosis. Access to nuclear DNA is required but not reachable in arrested cells. Viral replication intermediates gain access to the nuclear DNA when the nuclear membrane is disrupted during mitosis (162).

Insertional mutagenesis

When transcriptional elements contained in the retrovirus activate a nearby cellular oncogene through effects on promoter, enhancer or posttranscriptional regulatory elements (Figure 5A–C), the host cell may undergo malignant transformation. Pioneering studies of ALV-induced bursal lymphomas showed that the majority of the

tumors contains a provirus integrated in the vicinity of the c-*myc* proto-oncogene and express high levels of c-*myc* (163). Because retroviral integration is monoallelic (occurs in only one copy of a chromosome pair), inactivation of a tumor suppressor gene is far less frequent (159) (Figure 5D).

Gene activation by retroviral integration

When a provirus integrates near a gene controlling growth and alters its expression, the host cell may have a selective growth advantage (154). Such a cell will clonally

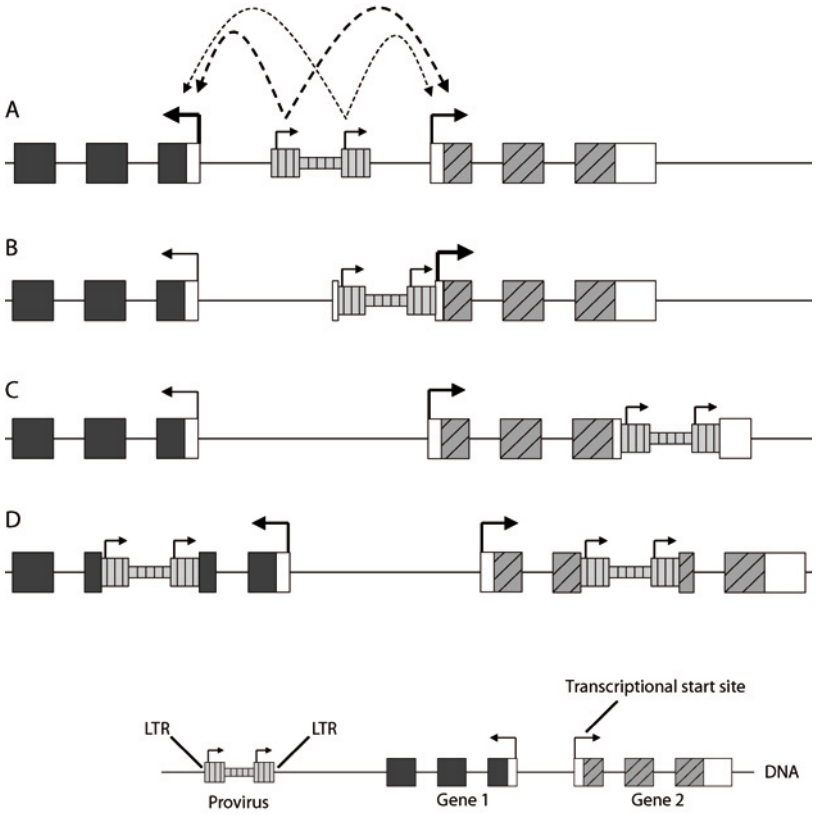

Figure 5. Schematic illustration of retroviral insertional mutagenesis. Different modes of proviral activation of Gene 1 and Gene 2 in hypothetical loci in the host cell genome. The unfilled parts of the first exons of the genes represent promoter regions. Integrating proviruses are depicted with two long terminal repeats (LTRs). Arrows denote transcriptional start sites. The thickness of the arrows represents levels of induced expression. A. Viral enhancer activation. The 5' LTR to the right usually provides a stronger enhancer effect than the 3' LTR. B. Viral promoter insertion activation. Either of the LTR promoters can drive transcription of the target gene. When the 5' LTR is used, splicing of viral sequences onto a target gene exon can sometimes be observed. C. Post-transcriptional dysregulation. Activation by enhancement from loss of potential RNA-destabilizing motifs. D. Gene truncation (gene I) or insertional activation (gene II). Different transcripts are likely to occur (not shown). Enhancer functions of viral LTRs are belived to decrease with increasing distance but are most likely active in the cases B-D as well. Adapted from (159)

expand and be enriched in a tumor population. The time required for this event as well as the requirement for additional genetic changes influences the latency period required for transformation by this mechanism. Cloning of this provirus and adjacent cellular DNA can identify this gene. If different tumors are compared, one could be able to find common insertion sites (CIS), repeatedly targeted by proviruses and more likely to harbor genes involved in tumorigenesis. The described scenario is normally a rare event and requires that enough cells become infected, in practice a sufficient virus titer.

The mode of retroviral integration has been debated as human immunodeficiency virus (HIV) was reported to favor integration "hot spots" in the genome (164). This issue was further addressed in a comparative study on murine leukemia virus (165). A distinct pattern of integrations could be found. Most often MLVs were integrated near transcriptional start sites of actively expressed genes in the genome. However, no hot spot regions could be found even for HIV, which integrated within transcriptional units somewhat randomly over the genome. A follow up report investigated the non-selected MLV integrations in detail (166). With the criteria used to define common insertion sites in many high-throughput screens to find cancer-causing genes, several CIS were found from non-selective integrations. In this way nearly two thirds of genes within a CIS with only two MLV integrations can be explained by natural retroviral site biases (based on 1200 integrations). Similarly, 20% of CIS with three MLV integrations would be false. However, CIS with more than 4 integrations would seldom be false positive here. There are other statistical approaches to find CISs from a substantial number of retroviral integration sites in noisy and biased environments (167). Such sophisticated methods correct for the increased probability of finding false CISs and have recently been used in a multidimensional analysis to indicate the presence of several cooperating oncogenes in retroviral screens (168).

High throughput screens to find cancer causing-genes

To reveal sites repeatedly targeted and avoid biases of too few samples, a large amount of tumors have to be screened. The almost complete information of the mouse genome sequence and rapid methods for cloning render high-throughput screening of hundreds of tumors possible. Several examples of such extensive experiments identifying genes in more than 150 CISs have been reported in mouse models of lymphoma and leukemia (169–171). A few studies describe viruses in human anogenital and liver carcinomas (172, 173) and suggest that identified proviral integration sites are not a coincidence.

The first high-throughput screens using retroviruses carried out in models of solid tumors was the tagging of glioma-causing genes (174) in our MMLV/PDGFB brain tumor model. According to current genome sequence databases, it identified 66 common insertion sites. A common insertion site was defined as a site where two, three, or more than three insertions were located within a maximum of 30, 50 and 100 kb, respectively. However, some previous studies have detected retro-

viral insertions in mouse mammary tumor virus-induced carcinomas (158). Most recently, a large screen identified 33 common insertion sites in candidate breast cancer oncogenes in MMTV-induced mouse carcinomas (175). In this paper, all tagged genes in MMTV and Moloney murine leukemia virus-induced malignancies (including the MMLV/PDGFB-induced gliomas) were compared to show that both viruses target mostly different genes suggesting distinct cancer pathways in different tumor types. A new promising technique to screen for cancer-causing genes in solid tumors involves the use of retrotransposons (176, 177). Sleeping Beauty transposons act as somatic insertional mutagens and have identified common insertion sites in various somatic tissues (reviewed in (178)).

Most screens of these candidate cancer genes have been published in the retroviral tagged cancer gene database (http://rtcgd.ncifcrf.gov) (179). The database supplies the majority of the retrovirally tagged genes published so far. It is updated frequently and also contains the majority of genes targeted by the retrotransposon tagging technique.

Mouse models of human cancer

Differences between human and mouse tumors

When mimicking human brain tumors in mice it is important to consider the existence of species differences. About 30% of laboratory rodents as well as humans have cancer in the end of their lifespan (2–3 years and 70–80 years respectively). However, humans undergo about 10^5 more cell divisions in a lifetime because they are 3000 times larger and live longer (180). Thus, development through evolution has created a greater intrinsic antineoplastic protection for humans. Some differences could be explained by the seven times higher metabolic rate and the poorer capacity of the liver to neutralize many carcinogens found in the mouse (181).

Fewer alterations of oncogenes and tumor suppressor genes are required in mice to transform cells into neoplasm *in vitro* and *in vivo* (182, 183). Furthermore, *in vitro* culturing of murine cells will often lead to spontaneous immortalization (184). This could in the mouse cells partly be explained by an overall higher level of telomerase, that enzymatically lengthens the telomeric ends. Here, the telomeres are substantially longer, preventing cells *in vitro* to enter replicative senescence that normally occurs in human cells, at late passages. The contribution of the p53 tumor suppressor pathway to human cell senescence is minor compared with its central role in murine cells. In human cells INK4A is the key governor of senescence instead (185). Despite of these differences several improved murine models of human tumors have been generated that could be exploited to understand how human cancers arise. By using the laboratory mouse several putative human oncogenes have been found. Relevant mouse models are important tools in evaluating better anti-tumor therapies.

Brain tumor models

Besides conventional transgenic/knockout models or xenotransplants of brain tumor cells, several brain tumor models mimicking sporadic tumor formation are now available (Table 1). In the mouse models, somatic mutations can be induced in a tissue specific and time-controlled fashion (186). For induction of such tumors either conditional genetically engineered models or somatic cell gene transfer can be used. In both, a normal microenvironment is able to respond to and interact with the transformed cells which also occur in the naïve disease. With a few exceptions, conventional knockouts and transgenic models with an initiating mutation present in all cells of the body are more suitable to mimic cases of familial brain tumors (or when looking at cooperating events for tumor progression).

A number of studies on somatic cell gene transfer have dealt with retroviral gene delivery with replication competent ALV splice acceptor (RCAS) vectors. These vectors with an inserted oncogene of interest are used to infect transgenic mice expressing the *t-va* receptor under the control of a cell-specific promoter (203). Two published promoters available in RCAS/TVA glioma models are for glial fibrillary acidic protein (GFAP) expressed by most astrocytes or nestin (Nes) expressed in immature cells like neuroepithelial stem cells and glial progenitors. Transgenic models or the use of other type of viral vectors, perhaps in combination with Cre recombinase inducible systems, are used in other glioma models. Several oncogenes and/or suppressor genes previously found in human gliomas have been used to induce gliomas of both low and high grades in these systems (Table 1).

Glioma models involving PDGF

Malignant neoplasms resembling human GBMs or primitive neuroectodermal tumors (PNETs) has been induced by a recombinant moloney murine leukemia virus encoding the PDGF B-chain (198). A combination of autocrine growth stimulation and insertional mutagenesis is believed to generate these tumors. Consistent nestin expression suggests an origin from an immature neuroglial progenitor. Decreased latency in the generation of malignant GBMs was found when Trp53 and Ink4a/Arf null mice were used for virus injection (204).

In the RCAS/TVA system, PDGF autocrine stimulation induced oligodendrogliomas and oligoastrocytomas from neural progenitors and astrocytes in wild type mice (191). Infection of *Ink4a/Arf* null mice with the same PDGFB virus generated more malignant high-grade tumors. By removing inhibitory regulatory elements in the PDGFB mRNA, protein expression can be substantially increased. Such a short form of PDGFB was expressed using the RCAS/TVA system and glial tumors with shortened latency, increased cellularity, regions of necrosis, and general high-grade character developed (205). This supports the view that PDGF have dose-dependent effects in gliomagenesis.

This shorter PDGF construct was recently used in a model where retrovirus was injected in the subcortical white matter of adult rats (*140*). Gliomas of high grade

Table 1. Examples of murine models of human gliomas.

Tumor[1]	Suppressor gene[2]	Oncogene[3]	Cell affected	Reference
AII		V12Ha-*Ras*	GFAP positive	Ding (187)
	Nf1+/- and *p53*+/- [4]		all cells	Reilly (188)
		v-src	GFAP positive	Weissenberger (189)
OII	*Arf-/-*		all cells	Kamijo (190)
		PDGFB	nestin positive	Dai (191)
		v-erbB	S100b positive	Weiss (192)
		V12Ha-*Ras* *EGFRvIII*	GFAP positive	Ding (193)
OAII	*Ink4a/Arf*+/+ or *Ink4a/Arf*-/-	*PDGFB*	GFAP positive	Dai (191)
		V12Ha-*Ras* *EGFRvIII*	GFAP positive	Ding (193)
AA		V12Ha-*Ras*	GFAP positive	Ding (187)
		V12Ha-*Ras* Ad:*EGFRvIII*	GFAP positive	Wei (194)
	Pten$^{f/f}$	V12Ha-*Ras*	GFAP positive	Wei (194)
	Nf1+/- and *p53*+/- [4]		all cells	Reilly (188)
		v-src	GFAP positive	Weissenberger (189)
	pRb, p107, p130		GFAP positive	Xiao (195)
	Ink4a/Arf-/-	*EGFR*	astrocyte or progenitor cells	Bachoo (144)
	PyV-mT		GFAP positive	Holland (196)
AO	*Ink4a/Arf-/-*	*PDGFB*	nestin positive	Dai (191)
	Ink4a/Arf-/- *or p53-/-*	*v-erbB*	S100b positive	Weiss (192)
GBM		*K-Ras* + *Akt*	nestin positive	Holland (197)
		PDGFB	mixed brain cells	Uhrbom (198)
		PDGFB	adult progenitors	Assanah (*140*)
	Nf1+/- and *p53*+/- [4]		all cells	Reilly (188)
	Nf1+/- and *p53*+/-[5]		GFAP positive	Zhu (199)
	Arf-/- or *Ink4a/Arf-/-*	*K-Ras*	nestin or GFAP positive	Uhrbom (200, 201)
	Ink4a/Arf-/-	*Ros*[5,6]	mixed brain cells	Charest (202)

[1] tumor type repeatedly found; [2]deleted or inactivated; [3]overexpressed or activated; [4] in *cis*; [5] in Cre-expressing cells; [6]*Ros*: ROS fusion tyrosine kinase, FIG-ROS; AII: diffuse low-grade astrocytoma (WHO II); OII: oligodendroglioma; OAII: oligoastrocytoma; AA: anaplastic astrocytoma (WHO III); AO: anaplastic oligodendroglioma (WHO III); GBM: glioblastoma multiforme; *Nf1*: Neurofibromin, type I; *PyV-mT*: Polyomavirus middle T antigen; *pRb*: Retinoblastoma; *Kras*: Kirsten rat sarcoma viral oncogene; *v-src*: Rous sarcoma virus; Ad: Adenovirus; *f/f*: flox/flox inactivation of gene from Cre recombinase expression.

developed only 14–20 days after injection but only 20% of cells in the brain tumors had been infected. This suggests that PDGF caused a massive expansion of both infected and uninfected glial progenitors via autocrine and paracrine stimulation.

Glioma-like lesions were also induced as PDGF-AA was infused in the SVZ of adult mice (29). PDGFRα positive stem cells (B cells) were targeted and stimulated to proliferate. The cells showed an addiction to the growth factor as their proliferation ceased when PDGF-AA delivery was stopped.

Glioma models involving other cancer genes

In the RCAS/TVA model, transfer of polyomavirus middle T antigen was found to generate high grade astrocytomas (196). Neither activated Ras nor activated Akt alone was found sufficient to induce gliomas. Only the combination of theses two proteins induced glioblastomas in nestin- but not in GFAP-expressing cells (197).

A transgenic model of activated Ras (V12-Ras) gave rise to rapidly growing malignant astrocytomas without Akt activation (187). Here, GFAP-expressing astrocytes were targeted and alterations in $p16^{Ink4a}$ and $p19^{Arf}$ were found as well as high levels of CDK4 and MDM2. Possibly, mutant Ras (in (187)) is sufficient to induce tumors due to expression (and tumor initiation) during embryogenesis as compared to the mice in RCAS/TVA model (197) where the expression of Ras is induced postnatally. A recent report shows that additional Pten inactivation in the transgenic Ras model will potentiate high grade glioma formation (194).

Kras activation and Ink4a/Arf loss may act together in gliomagenesis since these alterations generated GBM in both astrocytes and progenitor cells (201). Increased malignancy were found from Pten depletion in a model where inactivation of the suppressor gene *pRb* (together with *p107* and *p130* inactivation) generated astrocytomas from GFAP expressing cells (195). EGFR activation and Ink4a/Arf loss have been found to generate gliomas from differentiated astrocytes (144).

To mimic brain tumors developed in patients with mutated *NF1* (neurofibromin 1), transgene mice lacking *Nf1* was crossed with heterozygous *Trp53* knock out mice. Mice that harbored *Nf1* and *Trp53* (both genes located on chromosome 17) in cis position were selected. They developed tumors with features of astrocytoma progression (from grade II to grade IV) after inactivation of the wild type allele by LOH (188).

From these models several roles of the genetic mutations found in human gliomas have been confirmed. Further, several of these models could prove useful to assess therapeutic strategies to treat human tumors. For this purpose, bioluminescence imaging could be used to monitor tumor progression. In for example a genetically engineered RCAS/TVA glioma model (206) the bioluminescence correlates with the number of tumor cells because the gene encoding luciferase is controlled by the human E2F1 promoter. In these mice, the E2F1 promoter mediates tumor-selective expression probably as a result of loss of the pRb pathway.

Cancers arising in the conditional mouse models described above are histologically and genetically accurate. The models are however critizied by people using

xenografed tumor models for being less heterogeneous than the clinical disease. This is because the genetically engineered mouse tumor is often caused by a single (or a few) genetic lesion(s). If these models are used for drugs that target such a particular lesion, they might yield exciting results. That might of course not necessarily be translated into the clinic. For example, activating mutations of Ras, often used in the mouse models, are seldom found in human gliomas. By contrast, cells in xenografted models have the disadvantage of being exposed to selection pressures when cultured *in vitro*. In addition, these tumor cells do not have an environment of stromal components, inflammatory cells and vasculature represented in the original tumor from the patient.

Screening for candidate brain tumor genes

High-throughput retroviral tagging, as a means of identifying candidate cancer-causing genes, has almost entirely been studied in hematopoietic tumors. A PDGF-encoding retrovirus was used to initiate malignant brain tumors 13–42 weeks after intracerebral injection into newborn mice (174, 198) . The long latency period to generate these tumors probably reflects the inability of MMLV/PDGFB to transform cells directly and the need for multiple cooperative changes to induce the tumors. The underlying hypothesis was that tumors were generated by autocrine growth stimulation and proviral insertional mutagenesis.

From 108 brain tumors, 647 proviral integrations gave 66 common insertion sites (174) (CIS; loci targeted in two or more tumors). These CIS were termed Brain tumor loci (Btl). The list of all MMLV/PDGFB-tagged genes is available in the retroviral tagged cancer gene (RTCG) database (http://rtcgd.ncifcrf.gov) (179).

Several genes had previously been found to be associated with transformation or oncogenesis, providing proof of concept of the model of retrovirally induced gliomagenesis. These Btl genes were *Ddr1*, *Trp53*, *Fancc*, *Rad51l1*, *Eef1a1*, *Gli1*, *Fos* but also *Ccnd1* (see the RTCG database). Whereas the case for these genes in neoplasia is strong, the majority of genes tagged encode proteins with previously unknown involvement in malignancies or PDGF signaling. Strikingly, the entire family of nuclear factor I (NFI) DNA-binding protein genes were tagged by eight integrations (in total). Other genes belonging to protein families, like the presenilin-like proteases, *Sppl2b* and *Sppl3*, and two members of Rhesus family of ammonium ion transporters, *Rhbg* and *Rhcg*, were also tagged. The role of these gene families in transformation and oncogenesis remains to be elucidated.

The clinical trials of gene therapy for X-linked severe combined immunodeficiency (XSCID) patients further highlights the oncogenic potential of retroviral tagging (207, 208). Eight out of eleven children were successfully treated but three developed clonal T cell leukemias. In these three cases tumorigenesis was caused by insertional mutagenesis as vector integration occurred within the LMO2 locus, which codes for a known human T cell oncogene. These drawbacks of gene therapy have forced researchers to generate better gene therapy vectors that are less prone

to cause insertional mutagenesis. As retroviral insertional mutagenesis that easily could generate cancer in a few human patients, demonstrate how effective this technique is.

Most of the brain tumors induced by MMLV/PDGFB resembled human glioblastoma multiforme (GBM) but also a number of oligodendrogliomas of grade II and III were found. MMLV/PDGFB-injected wild type mice that developed oligodendroglioma-like tumors of lower grade had a prolonged survival in comparison to mice that developed GBM.

Expression analysis using multigene cDNA arrays confirmed several similarities of the mouse gliomas with human brain tumors (209). Both known and novel genes that could be involved in progression or act as putative tumor markers of the tumor types were found to be differentially expressed. Array and quantitative real-time PCR (qrt-PCR) analysis also revealed a similar profile of *Pdgfr*α and the retrovirally tagged genes *Abhd2*, *Ddr1*, *Fos, Ng2, Ppfibp1*, *Rad51B* and *Sulf2* displaying increased expression in normal newborn brain and highly elevated expression in the brain tumors compared to normal adult brain. Other Btl genes, like *Plekhb1*, *Prex1*, *Prkg2*, *Sox10* and *1200004M23Rik* were highly upregulated in the tumors but had a different expression profile than *Pdgfr*α. Finally, *Rap1gap*, *Gli*, *Neurl* and *Camk2b* were downregulated in the tumors compared to normal brain.

Concordant with previous studies, *Pdgfr*α expression was highly expressed in both mouse oligodendrogliomas and GBMs although the level of expression varied between individual tumors. When PDGF overexpression instead was specifically restricted to nestin-expressing neural progenitors and glial fibrillary acidic protein-(GFAP) expressing astrocytes, exclusively oligodendrogliomas of both low and high grade were reported (191). This finding indicates that PDGF overexpression without additional spontaneous or insertional mutations yields low-grade oligodendrogliomas. One might then argue that the long-latency, slowly growing tumors of the current study develop through a similar mechanism. Interestingly, significantly more insertions in Btls were found in early (fast growing) tumors than in the long-latency slowly growing tumors. Worth mentioning is that 5–10% of MMLV/PDGFB-infected mice lacking *Trp53* or *Ink4a/Arf* and 40–45% of the wild type mice did not develop any brain tumors (204).

In transgenic mice with an overexpressed PDGFB gene driven by the myelin basic protein (MBP) promoter, hypercellularity of oligodendrocyte precursors were found, but no brain tumors (210). By contrast, an elevated PDGFB expression caused by removing inhibitory elements in its mRNA could after *i.c.* injection after birth induce glial tumors of a higher grade of malignancy (205). Apparently, the levels of PDGF expression for transformation and progression are important in this system.

In addition, the expression analysis of the brain tumors showed that genes and proteins of the oligodendrocyte lineage were frequently activated. Nestin, Sox10, Olig2 and Ng2 were only a few examples of genes that are known to be expressed in OPCs that were elevated in both mRNA and protein levels.

A recent investigation of gene expression in neural stem cells (using a similar

cDNA microarray set) compared stem cells treated with FGF-2 kept in a dedifferentiated state with stem cells induced for differentiation in the absence of growth factor or from PDGF-AA stimulation alone (211). Intriguingly, when the transcriptional profile of FGF2-treated stem cells was compared with the profile of MMLV/PDGFB induced brain tumors a good correlation of gene expression was revealed. In addition, the majority of genes overexpressed in early GBM-like tumors were downregulated in differentiated neural stem cells. Moreover, the results sorted out genes that were differentially expressed in malignant PDGF signaling in the brain tumor cells from genes expressed in normal stem cells.

It is tempting to speculate about the origin of the mouse brain tumors and the cancer stem cell theory. For example we found several tagged genes that have a direct role in stem cell state maintenance. Besides the tagged genes *Gli1* and *Trp53* that are known regulators of normal or cancer stem cell renewal (212, 213), *Ncor2*, a gene tagged by five MMLV/PDGFB integrations, were recently reported to inhibit gliogenesis and keep neural cells in a stem cell state (214).

So far two Btl genes have been reported in further studies of glioma development and progression. Using the RCAS/tva system (described above) the GAP domain of P190RhoGAP have been found to decrease PDGF-induced brain tumor formation (215). *P190RhoGAP* which was targeted by retroviral integrations (within the gene) in three of the MMLV/PDGFB-induced mouse gliomas (174) showed a tumor suppressing role by inhibiting Rho activity. Similarly, the Sox10 gene, tagged five times (with all integrations located upstream of the gene) in the MMLV/PDGFB-induced gliomas, was found to enhance PDGF-induced brain tumor formation (by using the RCAS/tva model) and shown to have a broad distribution in human gliomas (216).

The roles of several other Btl genes in glioma formation are currently investigated. For example, *Prkg2*, that encodes cGMP-dependent protein kinase II, cGKII, was targeted by retroviral insertions in two MMLV/PDGFB-induced brain tumors. Different human glioma cells have been transfected with *Prkg2* and an overall reduction in colony formation and cell proliferation has been found, compared to controls transfected with truncated *Prkg2* (with the first 10 exons) or empty vector (unpublished observations).

Conclusion and future perspectives

The approach to retrovirally tag candidate brain tumor-causing genes from PDGF-encoding acutely transforming retroviruses turned out to be successful. Thus, we were able to present 66 brain tumor loci harboring candidate genes that could cooperate with PDGF in gliomagenesis (174).

The glioma model was evaluated with multigene cDNA arrays (209). Several differentially expressed genes were found when tumors were compared to normal brain. Among genes most likely to be differentially expressed, some genes belonging to a Btl, as well as several genes identified in human high-grade gliomas were

found. Early (fast growing) tumors were generally GBM whereas late (slow growing) tumors were generally diagnosed as oligodendrogliomas. In addition, genes involved in regulating progression and markers for these different glioma types were suggested.

A finding that strengthens the concept of insertional mutagenesis as a causative event in the MMLV/PDGFB glioma model is that the number of integrations correlates with the glioma development and progression. Hence, significantly more insertions were found in the high grade GBM tumors with short latency as compared to low grade long-latency oligonderoglial tumors (209).

The validation of the candidate cancer-causing genes identified has only just begun. First, genes unrelated to transformation targeted by coincidence or involved in viral replication must be sorted out and discarded. As enhancer mechanisms are known to operate over large distances (217) some of the loci were reported to tag more than one gene. Examination of expression of the tagged genes in the tumors where they were found could be important in order to select out the relevant ones.

Are the candidate genes identified with the MMLV/PDGFB glioma model relevant also in human brain tumor development? Expression analysis of the MMLV/PDGFB-induced brain tumors identified several differentially expressed genes previously found with a similar expression in human glioma samples. PDGF is a mitogen for both mouse and human glial cells and executes similar downstream signaling pathways in both species. Mouse cells are more easily transformed as compared to human cells in part because of their increased levels of telomerase in their cells. Normal human astrocytes require at least four genetic alterations (hTERT expression, Ras pathway activation, p53 inactivation, and pRb/p16 pathway inactivation) for astrocytoma formation (218). Apart from hTERT expression (which is already upregulated in mice), the other three pathways have all been implicated in mouse models of human gliomas (see previous chapters).

Expression analysis of corresponding candidate genes in the human disease has to be performed and compared with their expression in mouse gliomas. Both mRNA and protein expression of selected Btl genes have to be analyzed and studied in human brain tumors samples and cell lines.

Loci encoding microRNAs have been frequently targeted in the MMLV/PDGFB model. Some of the microRNAs targeted are now being evaluated. The expression of a cluster of miRNA29a and miRNA29b in brain tumor cells is investigated. These microRNAs are close to five proviral integrations in a locus on mouse chromosome 6 with very few genes or expressed sequence tags. A recent report shows that microRNA29a and microRNA29b were specifically up-regulated in mouse tumors containing retroviral integrations close to these microRNAs (219). This finding potentiates their role in leukemogenesis. MicroRNA-21 previously found to be overexpressed and anti-apoptotic in glioma cells was also tagged in three brain tumors; in one tumor less than 2 kb upstream of the mature microRNA. Particularly, several insertions in retroviral tumor screens (like ours) are close to microRNAs (as reported in (220)). This phenomenon could correlate with the fact that the retroviruses often integrate near chromosomal fragile sites (221) i.e. regions that have an overrepre-

sentation of microRNAs (75). About a hundred fragile sites have been found in the human genome. They are regions found to be related to cancer development as they are susceptible to chromosome breakage and to amplifications (222).

Some of the tagged genes are especially interesting as they belong to the same protein families and thus could affect similar downstream signaling pathways. For example, genes of *Sppl2b* and *Sppl3* that belong to the family of presenilin-like proteases and the entire family of nuclear factor I (NFI) DNA-binding protein genes were tagged. As *Nfia*, *Nfib* and *Nfic* each were targeted once; *Nfix* was tagged in five different mouse gliomas. Similarly, three integrations downstream of both *Rhbg* and *Rhcg* genes were found. These genes encode proteins that belong to the Rhesus family of ammonium ion transporters. However, the microRNAs *mmu-mir-9-1* and *mmu-mir-9-3* that give rise to an identical mature miRNA sequence are also located downstream of *Rhbg* on chromosome 3 and *Rhcg* on chromosome 7 in the mouse genome, respectively. It is then tempting to suggest that it is rather these microRNAs that are retrovirally tagged in these brain tumors.

As described above, seven Btl genes had previous implications in tumorigenesis. Several other Btl genes have after the time of publication been reported to have a functional role in tumorigenesis; *Tax1bp2* was tagged in six of the MMLV/PDGFB-induced mouse brain tumors. A recent study describes that downregulation of *Tax1bp2* expression by siRNA induces centrosome hyperamplification (223) which results in aneuploidy, often seen in cancers. Although *Sulf2* (tagged in two mouse gliomas) is upregulated in breast cancer (224) as well as in our MMLV/PDGFB tumors (209) it has been reported to have a suppressor role in myeloma (225). *Rap1gap* that was tagged three times has been found to suppress tumor cells in different types of human cancer (226, 227). Downregulation of the gene contributes to Ras transformation (228). *Btg2* has been found to have a major role during p53 suppression of Ras transformation (229). In the same intron as *Camk2b* was tagged in MMLV/PDGFB-induced gliomas it was also targeted by insertional mutagenesis in a recent gene therapy model screening for genes involved in T-cell transformation (230). *Map2k5* (tagged in five mouse gliomas) has been found to induce apoptosis if overexpressed in medulloblastoma cells (231). Although the majority of reported genes targeted by retroviral insertional mutagenesis are putative oncogenes, a lot of genes identified in the MMLV/PDGFB model have been reported to function as putative tumor suppressors. A reason for the possible targeting of a greater fraction of suppressor genes in the MMLV/PDGFB model might reflect the use an oncogene, PDGFB to initiate tumorigenesis.

Moreover, elevated expression of a number of Btl genes implicate that they may have a role in brain tumors: *Plekhb1* has been found to differ in a screen where pilocytic astrocytoma was compared to glioblastoma (232). Expression of *Sox10* has been implicated to be a ubiquitous marker for glioma cells (216, 233, 234); The Btl gene *Sdc3* is also found to be expressed in glioma cells (235).

Three integrations in each of *Ddr1* and *Cspg4* (NG2) genes were found. *Ddr1,* a tyrosine kinase with a known role in gliomagenesis has further been found to promote glioma cell invasion (236) and correlates with a poor prognosis in glioma

patients (237). *Cspg4* overexpression has been known to increase tumor initiation and growth rates, neovascularization, and cellular proliferation in gliomas (238).

Frequently tagged genes identified in our screen like *Nfix* and *Rad51l1* were also found among the relatively few integrations found in a screen for imatinib-resistance genes in a mouse model of chronic myeloid leukemia (239). This indicates that several of the tagged genes in the MMLV/PDGFB model are not only involved in PDGF signaling but might also participate in imatinib (and thus PDGFR treatment) resistance.

In addition, of the consensus sequences recently discovered with mutations in colorectal and breast cancer patients at least four Btl genes were present including, *TP53*, *RAP1GA1*, *SULF2* and *GLI1* (240). All these examples of tagged Btl genes that have been proposed a role in tumorigenesis potentiate the relevance of our findings. A further role for the proteins of these retrovirally tagged glioma genes in PDGF signaling must be elucidated.

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