A Short Thesis about Growth Factors in Gliomas

BY

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Abstract

Glioblastoma multiforme (GBM) is the most common form of primary brain tumor in humans. Its aggressive and infiltrative growth into the brain, and, at best, only partial sensitivity to radiotherapy and chemotherapy, renders it extremely difficult to treat and survival remains dismal.

Growth factors, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), and their corresponding receptors are seen in glioma tissue, suggesting the presence of autocrine stimulatory loops. PDGFβ and a mutated EGF receptor were also identified as cellular homologues of two oncoviruses, thereby indicating a role in tumorigenesis. This thesis presents a brain tumor model in mice, developed using a PDGFβ coding retrovirus to induce overexpression of PDGF-B in neonatal mouse brain. Immature tumors, with histological characteristics of primary brain tumors developed at relatively high frequencies. Mice injected with a non-coding retrovirus did not develop tumors, indicating the crucial role of PDGF stimulation in this system. Tumor cells were also shown to continue to depend on PDGF stimulation when cultured in vitro.

In human glioblastomas, growth factor receptor signaling is present in conjunction with defects in cell cycle arrest pathways. When the PDGFβ-virus model was used with p53 or Ink4a-Arf deficient mice, tumors arose with shorter latency and higher frequency. Loss of p53 or Ink4a-Arf seemed to facilitate signaling through the PI3K/Akt pathway. Thus, a functional role for the co-existence of p53 loss of function and PDGF signaling in a subset of gliomas is presented.

Human GBM samples were collected and analyzed with respect to expression and activation of the EGFR and PDGFRα. Most tumors expressed the both receptors at moderate to high levels, but high activation of either receptor seemed mutually exclusive.

Keywords: glioma, mouse model, PDGF, EGFR, activation

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PAPERS INCLUDED IN THE THESIS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:


III Hesselager Göran, Uhrbom Lene, Westermark Bengt, Nistér Monica. Complementary effects of platelet-derived growth factor (PDGF) autocrine stimulation and p53 or Ink4a-Arf deletion in a mouse glioma model. (In progress)

IV Hesselager Göran, Ren Zhi-Ping, Saft Leoni, Hägerstrand Daniel, Wester Kenneth, Östman Arne, Nistér Monica. Different glioblastoma multiforme subgroups exist with regards to EGFR and PDGFRα activation. (Submitted)
### Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>ARF</td>
<td>Alternative reading frame protein</td>
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<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
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<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<tr>
<td>GRP</td>
<td>Glial restricted precursor</td>
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<tr>
<td>INK4a</td>
<td>Inhibitor of CDK 4</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>MDM2</td>
<td>Murine double minute 2 protein</td>
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<tr>
<td>MEK</td>
<td>Mitogen-induced extracellular kinase</td>
</tr>
<tr>
<td>MMLV</td>
<td>Moloney murine leukemia virus</td>
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<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<tr>
<td>NF1</td>
<td>Neurofibromatosis type 1 protein/gene</td>
</tr>
<tr>
<td>OPC</td>
<td>Oligodendrocyte precursor cell</td>
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<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
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<tr>
<td>PDK</td>
<td>PI3K dependent kinase</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3’-kinase</td>
</tr>
<tr>
<td>PLCγ</td>
<td>Phospholipase C-gamma</td>
</tr>
<tr>
<td>PNET</td>
<td>Primitive neuroectodermal tumor</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase with homology to tensin</td>
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<tr>
<td>RB</td>
<td>Retinoblastoma protein/gene</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming growth factor alpha</td>
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<tr>
<td>TSC</td>
<td>Tuberous sclerosis complex</td>
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Background

Introduction
The most common primary tumor of the central nervous system in adults is glioblastoma multiforme (GBM), and unfortunately it is also the most malignant type. Growing fast and diffusely infiltrating normal brain tissue it is still considered incurable.

Relatively soon after growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) were discovered, they were also identified in high quantities in GBM cell lines and tissue. They were found not only to induce mitogenic and migratory responses, but were also able to transform cells in vitro. It was early hypothesized that uncontrolled growth factor stimulation could have a role in tumor initiation. As knowledge of growth factor receptor signaling and cell cycle control pathways have grown, it has become increasingly clear that this is the case. By mimicking certain aberrations seen in GBMs in mice, different models of gliomas have been created, thus, proving the importance of these alterations in gliomagenesis. By combining specific alterations/mutations in the mouse models, further support for a functional role of specific patterns of aberrations found in GBMs, can been gained.

If GBMs continue to depend on growth factor stimulation, the logical consequence would be to block their activation. The development of specific small molecule tyrosine kinase inhibitors with tolerable side effect profiles has made this a real possibility. As a result of intertumoral variations of receptor expression and activation, treatment results, in individual cases as well as in larger studies, may be obscured if these factors are not taken into account.

An overview of the network of proteins regulating cell cycle arrest and signal transduction pathways downstream of growth factor receptors will here precede the discussion of the factual changes seen in GBMs.
Normal cell functions

Cell cycle arrest pathways
The replicative cell cycle process is divided into four phases: G1, S, G2, and M. DNA synthesis/duplication occurs in S phase and chromosome separation and cell division (mitosis) takes place in M-phase. G1 is the gap-phase between M- and S-phase and G2 is the phase between S and M phase. During G1 the cell responds to extracellular cues and it is decided if the cell will progress through the cell cycle or enter a resting, quiescent stage, G0. The time in late G1 phase when the cell makes the decision to begin DNA synthesis is called the “restriction point” and passage beyond this point means that the cell is irreversibly dedicated to cell cycle progression and will complete the cell cycle with or without further mitogenic stimuli (Pardee, 1989). Passage through this G1 restriction point is closely guarded by an intricate system of protein interactions (Figure 1).

Several signaling pathways converge at the phosphorylation and inactivation of the retinoblastoma protein (RB). This is central in promoting G1-S transition, and conversely is RB activity itself essential for induction of cell cycle arrest replicative and senescence. RB functions as a tumor suppressor in several ways. First identified was its inhibitory binding to E2F transcription factors. It was later shown that RB, upon binding E2F, recruits histone deacetylases (HDAC) to form active transcriptional repressor complexes that binds to DNA (Harbour and Dean, 2000; Zhang et al., 2000; Zhang et al., 1999). Cyclin D activation of cyclin D-dependent kinase 4 or 6 (CDK4 and CDK6) leads to phosphorylation of RB (Kato et al., 1993; Matsushime et al., 1992; Weinberg, 1995). RB and HDAC then dissociate, resulting in a derepression of E2F target genes. If RB is further phosphorylated by CDK2 and cyclin E (later cyclin A), the E2F will dissociate from RB and is thereby free to transactivate target genes important for S phase entry (Harbour et al., 1999).

The Cip/Kip family of tumor suppressor proteins, p21Cip1 (Harper et al., 1993; Xiong et al., 1993), p27Kip1 (Polyak et al., 1994a; Polyak et al., 1994b), and p57Kip2 (Lee et al., 1995; Matsuoka et al., 1995) have their main role as inhibitors of the Cyclin E-CDK2 complex. The Cip/Kip proteins were earlier also thought to inhibit the cyclin D-CDK4/6 complex, but has now been shown to form a functionally active complex with these proteins. By being sequestered with cyclin D-CDK4/6, are they unable to inhibit the cyclin E-CDK2 complex (Cheng et al., 1999; LaBaer et al., 1997). Mitogen induced
upregulation of cyclin D seems to be rate limiting in the formation of active complexes with CDK4/6. If mitogens are withdrawn, cyclin D is quickly degraded, and Cip/Kip proteins earlier sequestered are now able to inhibit cyclin E-CDK2 and hamper progression (Sherr, 1993).

The INK4 proteins are another group of CDK inhibitors. The four known INK4 proteins are the original p16INK4a (Serrano et al., 1993), and closely related p15INK4b (Hannon and Beach, 1994), p18INK4c (Guan et al., 1994), and p19INK4d (Chan et al., 1995). The INK4a gene is often deleted or inactivated in many human cancers including glioblastomas (Hirama and Koeffler, 1995; Liggett and Sidransky, 1998). The protein exerts its function by binary binding of CDK4 or 6, thereby preventing RB phosphorylation and also releasing Cip/Kip proteins to inhibit cyclin E-CDK2 (Hannon and Beach, 1994; Serrano et al., 1993). Mice lacking the Ink4a protein specifically are more susceptible to tumor formation by other oncogenic stimuli, but do not develop spontaneous tumors (Krimpenfort et al., 2001; Sharpless et al., 2001).

The INK4a gene locus includes a second tumor suppressor gene, ARF (Kamijo et al., 1997; Quelle et al., 1995). With a different first exon and parts of exons two and three read in an alternative reading frame, the ARF gene is almost always affected in parallel with the INK4a gene. The p14ARF protein (p19Arf in mice, both referred to here as ARF) is structurally and functionally different from INK4a. It serves as a sensor of increased oncogenic/hyperproliferative stimuli from inducers of S-phase entry (Bates et al., 1998; de Stanchina et al., 1998; Palmero et al., 1998; Zindy et al., 1998) and has several roles in preventing S-phase entry. First discovered was ARF’s ability to prevent murine double minute 2 (MDM2) protein downregulation of p53 (Pomerantz et al., 1998; Zhang et al., 1998). MDM2 binding to p53 interferes with p53’s transactivation of target genes (Momand et al., 1992). MDM2 also functions as an ubiquitin ligase and targets p53 for degradation (Honda et al., 1997; Honda and Yasuda, 1999). Thirdly, MDM2 relocates p53 to the cytoplasm where it undergoes proteosomal degradation (Tao and Levine, 1999; Weber et al., 1999). ARF has also been identified to have p53-independent function (Weber et al., 2000). It can bind directly to E2F, leading to downregulation of E2F activity and inhibition of cell cycle progression (Mason et al., 2002).

The tumor suppressor protein p53 plays a central and very important role in cell cycle control and by protecting the integrity of the genome. The growth arrest mechanism of p53 depends on its regulation of G1 progression by transcriptional activation of p21Cip1 (Dulic et al., 1994; el-Deiry et al.,
It may also inhibit cell cycle progression in G2 by transcriptional activation of 14-3-3 sigma (Hermeking et al., 1997) and transcriptional repression of cdc2 (Taylor et al., 1999) and cyclin B1 (Innocente et al., 1999). Its apoptotic functions, in response to aberrant proliferation signals or DNA damage, are mediated by transcriptional activation of Bax (Miyashita and Reed, 1995), the gene for insulin-like growth factor binding protein, IGF-BP3 (Buckbinder et al., 1995), and several not yet verified target genes. The p53 protein may also induce apoptosis through transcriptionally independent mechanisms, involving proteins such as caspase-8 (Ding et al., 2000). The activity of p53 is partly autoregulated with p53 inducing MDM2 transcription (Barak et al., 1993) and inhibiting ARF transcription (Stott et al., 1998).

Simplified, the regulation of a cell’s passage through the G1 restriction point can be divided into two arms (Figure 1). One involves INK4a inhibition of the CDKs phosphorylation of RB. The other involving ARF’s prevention of MDM2 mediated downregulation of p53. The final effect of both pathways is the repression of transcription of E2F target genes (Rowland et al., 2002). These arms link into each other at several other points, forming a network. ARF may interact with E2F (Mason et al., 2002), MDM2 can bind to RB (Xiao et al., 1995), and ARF itself is upregulated by E2F activity (Dimri et al., 2000).

Growth factors, their receptors and results of receptor activation

There are many growth factors with corresponding receptors expressed in glioma tissue. The ones mainly implicated in glioma growth includes platelet-derived growth factor (PDGF) with receptors, the epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) both binding to the EGF receptor (EGFR). Insulin-like growth factor I (IGF-I) and basic fibroblast growth factor (bFGF) and their receptors also play a role in gliomas but will not be discussed in detail here. Growth factors are relatively small molecules, usually less than 30 kDa. The receptors are larger integral membrane proteins, around 160-180 kDa, and belong to the polypeptide growth factor receptor tyrosine kinase superfamily, which also includes the insulin receptor and c-kit among others. The receptors have in common an extracellular ligand-binding domain, a short helix formed transmembrane domain and a cytoplasmic protein tyrosine kinase domain. Upon ligand binding, receptors characteristically dimerize and each subunit transphosphorylates the other. This creates binding sites for intracellular molecules with intrinsic signaling functions and for formation of signaling complexes. The most intensively studied signaling pathways are the PI3K
pathway, the RAS/MAPK pathway and the PLC-γ pathway, which will be discussed below. The different tyrosine kinase receptors utilize to a large extent the same pathways, but like stimulation of the same receptor type can have different responses depending on circumstances, subtle variations in second messengers and the type or status of the cell most likely determine the specific cellular effects of the various receptors.

**PDGF/PDGFR**

PDGF was originally identified in serum and found to be a mitogen for cultured fibroblasts, smooth muscle cells, and glia cells (Kohler and Lipton, 1974; Ross et al., 1974; Westermark and Wasteson, 1976). The protein was purified from platelets, which store PDGF in their α-granules (Antoniades et al., 1979; Heldin et al., 1979). The PDGF family of growth factors consists of five disulfide-bonded homo- and heterodimeric isoforms; PDGF-AA, -BB, -AB, -CC, and -DD (Heldin et al., 2002; Heldin and Westermark, 1999). The different isoforms bind with different affinity to the two structurally related PDGF receptors, the PDGF α-receptor (PDGFRα) and the PDGF β-receptor (PDGFRβ). The PDGFRα binds the PDGF-A, -B, and C isoforms with high affinity, while PDGFRβ binds the PDGF-B and -D chains (LaRochelle et al., 2001; Li et al., 2000; Seifert et al., 1989).

The biological function varies between ligand isoform, receptor type, presence of other growth factors, and cell type stimulated. The normal functions *in vitro* include cell proliferation, chemotaxis, promotion of cell survival, and differentiation. Under normal physiological conditions, PDGF signaling plays important roles in embryonic development, wound healing, and vasculogenesis (Ataliotis and Mercola, 1997; Heldin and Westermark, 1999).

**TGF-α/EGFR**

The epidermal growth factor receptor (EGFR, also HER-1 (human EGF receptor 1) and c-erbB1) (Ullrich et al., 1984) is the most studied member in a family of four receptors including ErbB2 (Neu, HER-2) (Schechter et al., 1985), ErbB3 (HER-3)(Kraus et al., 1989), and ErbB4 (HER4)(Plowman et al., 1990). There are several ligands capable of binding to these receptors including EGF itself and transforming growth factor-alpha (TGF-α) (Derynck et al., 1984). As for the PDGFs, EGFR activation may result in a multitude of cellular responses, like mitogenesis, migration, differentiation and dedifferentiation, depending on the context. The EGF family of growth factors and receptors play a significant role during neural development (Meyer and Birchmeier, 1995).
Signaling pathways downstream of growth factor receptors

The most studied signaling pathways include the phosphatidylinositol 3’-kinase (PI3K)/AKT pathway, RAS/mitogen activated protein kinase (MAPK) pathway, and the phospholipase C (PLCγ) pathway (Figure 1).

**PI3K pathway**

PI3K consists of two subunits, p85 and p110, with the regulatory p85 subunit binding to the activated receptor and the p110 subunit containing the catalytic domain. Localization to the receptor brings the protein in proximity to the cell membrane where it specifically phosphorylates the D3 position of the inositol ring of phosphatidylinositides to generate phosphatidylinositol-3,4-biphosphate (PIP₂) and phosphatidylinositol-3,4,5-triphosphate (PIP₃). The tumor suppressor phosphatase with homology to tensin (PTEN), can dephosphorylate the D3 position of PIP₃, and thereby counter PI3K signaling (Stambolic et al., 1998). The membrane bound PIP₃ binds in turn to a member of the AKT family of serine-threonine kinases. The family consists of AKT-1, AKT-2, and AKT-3, having somewhat different functions, but will here be referred to as AKT. AKT is phosphorylated on Thr-308 and Ser-473 by a PI3K-dependent kinase (PDK) and PIP₃.

Further downstream, AKT has several different substrates that play important roles in cell growth, metabolism, survival, and mitogenesis. AKT phosphorylation of the tumor suppressor tuberous sclerosis complex-2 (TSC2) disrupts a TSC1-TSC2 heterodimer, relieving this complex’ inhibition of p70 S6 ribosomal kinase (S6K), perhaps by way of mammalian target of rapamycin (mTOR) and PDK1 (Inoki et al., 2002; Potter et al., 2002). This will result in inactivating phosphorylation of the translational repressor 4E-BP1 and thereby lead to increased protein translation and cell growth (Potter et al., 2002). AKT also potentiates hypoxia-induced factor 1-alpha (HIF1-α) expression resulting from hypoxic conditions or mitogenic signaling (Zundel et al., 2000). HIF1-α stability leads to transcription of vascular endothelial growth factor and thereby vasculogenesis (Forsythe et al., 1996; Mazure et al., 1997; Mazure et al., 1996).

Perhaps the most important function of AKT is the antiapoptotic activity of the protein. It inactivates the apoptosis promoting proteins such as BAD (Datta et al., 1997), FKHR (Brunet et al., 1999), and caspase-9 (Brunet et al., 1999) by phosphorylation. AKT phosphorylation of MDM2 leads to faster nuclear localization of MDM2, resulting in decreased levels of p53 (Mayo et al., 2002; Zhou et al., 2001) thereby inhibiting the apoptotic and anti-
Figure 1. Growth factor signaling and cell cycle arrest pathways
mitogenic functions of p53. Additional PTEN-p53 interactions are overviewed in the discussion of paper III below. Akt also leads to increased mitogenic activity by phosphorylation of p27\textsuperscript{kip} (Graff et al., 2000), and by phosphorylating glycogen synthase kinase-3β (GSK3β) and thereby preventing cyclin-D1 degradation (Diehl et al., 1998).

**RAS/MAP kinase pathway**

Activation of tyrosine kinase receptors leads to the recruitment of a complex of the adapter molecule Grb2 and the guanine nucleotide exchange factor, Sos, to the plasma membrane. This complex binds either directly or via the adapter protein Shc to the receptor. Sos may then activate the small G protein Ras by exchanging its GDP for GTP (Schlessinger, 1993). The gene coding for the tumor suppressor neurofibromin (NF\textsubscript{1}), lost in neurofibromatosis type 1, negatively regulates Ras by counteracting the activation of Sos (Martin et al., 1990; Xu et al., 1990). Once activated, Ras interacts with several effector proteins. Ras can activate PI3K, but the main signaling cascade follows activation of Raf, which in turn phosphorylates MAPK-kinase (MAPKK) also termed mitogen-induced extracellular kinase (MEK). MAPKK is of course an activator of MAPK, collective name for p38 and extracellular signal regulated kinase (ERK) 1 and 2. Activated MAPK translocates to the nucleus where it phosphorylates and activates several transcription factors important in a mitogenic response (Schlessinger, 2000). One critical function of RAS/MAPK signaling is induction of cyclin D expression, a rate limiting factor in promoting cell cycle progression (Aktas et al., 1997).

**PLC-γ pathway**

Another protein that binds directly to and is activated by most tyrosine kinase receptors is PLC-γ. Activation by phosphorylation makes PLC-γ hydrolyze phosphatidylinositol(4,5)P\textsubscript{2} into diacylglycerol (DAG) and inositol(1,4,5) phosphate (IP\textsubscript{3}) (Kim et al., 1991). DAG in turn activates protein kinase C (PKC) and IP\textsubscript{3} mobilizes intracellular Ca\textsuperscript{2+} (Berridge, 1993; Rhee and Choi, 1992). These events may result in migration of some cell types. PKC activation also plays an important role in the mitogenic and oncogenic response to receptor tyrosine kinase activation in certain cell types (Obermeier et al., 1996).

**Progenitor cells**

The development of astrocytes and oligodendrocytes is regulated by several of the same growth factors that are active in gliomas. From a tumor biology
perspective, glial progenitors are interesting, as they have been proposed to be the cells-of-origin for gliomas. GBMs are heterogeneous tumors in several ways, having both immature and differentiated areas, and may have areas of malignant astrocytic cells side by side with areas of oligodendroglioma histology. This implies that the cell-of-origin must be either a cell that can dedifferentiate or a progenitor cell with multi-, oligo-, or bipotential developmental capabilities. Historically, neurons and glial cells (astrocytes and oligodendrocytes) were thought to develop from different precursor pools that diverged early during embryonal development. Common for neuroepithelial stem cells as well as glial and oligodendrocyte precursor cells is that they express the intermediate filament nestin (Gallo and Armstrong, 1995; Lendahl et al., 1990). Originally thought to be specific for progenitor cells it has later been found in reactive astrocytes (Clarke et al., 1994), endothelial cells and GBMs (Dahlstrand et al., 1992; Tohyama et al., 1992).

During the last 20 years, evidence showing that common progenitor cells also appear late in development and in the adult brain has accumulated. Radial glia in the developing brain (Malatesta et al., 2000; Noctor et al., 2001; Noctor et al., 2002) and astrocyte-like cells in the mammalian adult sub-ventricular zone (SVZ) (Doetsch et al., 1999; Laywell et al., 2000) are able to give rise to both neurons and astrocytes.

Oligodendrocytes have been shown in different experiments to develop from neuroepithelial stem cells by way of glial restricted precursors (GRP) (Rao and Mayer-Proschel, 1997; Rao et al., 1998), as well as from cells that may also develop into motor neurons (Lu et al., 2002; Lu et al., 2000; Zhou et al., 2000). An earlier identified oligodendrocyte precursor cell (OPC) also called O-2A cell is supposedly an intermediate between the GRP and the differentiated oligodendrocyte. It may in vitro give rise to both oligodendrocytes and type-2 astrocytes (Raff et al., 1983; Raff et al., 1984), but the existence of type-2 astrocytes in vivo is uncertain (Espinosa de los Monteros et al., 1993). Characteristic for OPC is that they express the PDGFRα (Pringle et al., 1992) and can be kept in a continuous proliferating state in vitro by addition of PDGF together with bFGF (Bogler et al., 1990; McKinnon et al., 1990; Noble et al., 1988; Richardson et al., 1988).

The heritage tracing of CNS cells is very complex and confounded by several factors. For one, there exist several different subtypes within the major groups neurons, astrocytes, and oligodendrocytes, perhaps following varying developmental pathways. There are also indications that mature cells have the ability to de-differentiate in vitro as a result of mutations such as
Ink4a-Arf deletion (Bachoo et al., 2002) or exogenous factors such as growth factors (Dai et al., 2001), and in vivo (Uhrbom et al., 2002). It is also unclear if the cellular markers for a differentiated phenotype really are exclusive. There may be a subgroup of true progenitor cells within the pool of mature cells, all expressing the same antigens or intermediate filaments such as GFAP.

Gliomas

Histology and grading of diffuse gliomas
The term glioma indicates that tumors arise from glial cells or progenitor cells of the glial lineage. In this study, the focus will be on diffusely infiltrating tumors of the astrocytic and oligodendroglial lineage. Diffuse, low-grade gliomas (WHO grade II) are categorized into astrocytomas (AII), oligodendrogliomas, and mixed oligoastrocytomas, depending on the cells resemblance to astrocytes or oligodendrocytes. Astrocytomas have a high degree of cellular differentiation and show diffuse infiltration of neighboring brain structures. Moderately increased cellularity, occasional nuclear atypia, and no visible mitotic activity are characteristic for the histological picture. Lesions typically affect young adults (mean age of 34 years at first biopsy) and have an intrinsic tendency for progression to gliomas of higher malignancy grades. (Kleihues and Cavenee, 2000; Kleihues et al., 2002)

WHO grade III gliomas constitute a more aggressive form and include anaplastic astrocytomas (AA) and anaplastic oligodendrogliomas. They can arise without previously identified disease or from preceding grade II glioma. In most respects, AAs take up an intermediate position between AII and the most aggressive gliomas, glioblastoma multiforme (GBM, WHO grade IV). Patients are somewhat older (mean age 41 years at diagnosis) than AII patients, but younger than GBM patients (53 years). Histologically AAs have markedly increased cellularity, distinct nuclear atypia, and marked mitotic activity with plenty of visible mitoses (Kleihues and Cavenee, 2000; Kleihues et al., 2002).

GBMs develop de novo, primary glioblastomas, or through progressive malignification of lower grade gliomas, termed secondary glioblastomas (Scherer, 1940). Gliosarcomas and giant cell glioblastomas are two rare variants that also sort into the GBM diagnosis. Tumors are generally composed of poorly differentiated neoplastic astrocytes but may have
regions with relatively differentiated cells. They are histopathologically characterized by cellular polymorphism, nuclear atypia, brisk mitotic activity, vascular thrombosis, microvascular proliferation, and areas of necrosis. It is the presence of endothelial cell proliferation and necrosis that definitively set GBM apart from AA (Kleihues and Cavenee, 2000; Kleihues et al., 2002).

Etiology, standard treatment, and survival

GBM, like other forms of cancer, is a disease resulting from genetic changes. What in turn causes these changes is in the majority of cases unknown. The only undisputed known exposure or environmental agent to cause gliomas is therapeutic X-irradiation. Prophylactic CNS radiotherapy of children with acute lymphatic leukemia leads to an increased risk of developing AII, AA, and GBM (Edwards et al., 1986; Vowels et al., 1991). There are several inherited tumor syndromes which predispose for glioma formation. The Li-Fraumeni syndrome, caused by germ line mutations in the TP53 gene, is characterized by multiple primary tumors, mainly soft tissue sarcomas and osteosarcomas, but increased incidence of astrocytomas is also seen (Greenblatt et al., 1994). Turcot syndrome type 1, caused by genetic alterations in mismatch repair genes predisposes patients to glioblastomas relatively early in life (Hamilton et al., 1995). Neurofibromatosis type 1 (NF1), characterized by multiple neurofibromas, malignant peripheral nerve sheath tumors, and several types of gliomas, is caused by mutations in the NF1 gene (coding for the Ras-GTPase neurofibromin). The syndromes mentioned are caused by mutations that in some way affect the control of DNA integrity or cause increased oncogenic signaling.

There are regional variations in the world but their cause is unknown. North America and most parts of Western Europe have a yearly GBM incidence of about 2-3/100.000 (Davis, 1998).

Standard treatment in most of the Western world combines some degree of tumor resection or biopsy with radiotherapy, often followed by chemotherapy. Gross total resection of GBM has been shown to increase survival (Nitta and Sato, 1995), though this is still measured in weeks to months, rather than years. Radiotherapy is given to all GBM patients regardless of the extent of the operation. Chemotherapy consists of alkylating drugs such as carmustine (BCNU), combination of procarbazine, cisplatin, and vincristine (PCV), or temozolomide (Gaya et al., 2002). In spite of maximum treatment, median survival is still around 1 year. The
strongest prognostic factor seems to be age, with older patients having poorer prognosis (Reavey-Cantwell et al., 2001).

Molecular aberrations in gliomas

Typical for many malignancies including gliomas, especially GBM, is the combination of uncontrolled signaling downstream of growth factor receptors and disturbances in cell cycle control. In most normal cells, excessive signaling downstream of RAS is sensed by INK4a and ARF with p53 resulting in apoptosis or cell cycle arrest (Lin et al., 1998a; Serrano et al., 1997). Damage to these control systems for entry into S-phase will allow mitogenic and anti-apoptotic signals to play out their roles unabated and the uncontrolled cell growth (and migration) seen in tumors will ensue.

Signaling abnormalities in gliomas

In gliomas the hyperactive signaling is caused by production of growth factors such as PDGF, EGF, TGF-α, and IGF-I in parallel with (over)expression of their corresponding receptors, indicating a probable autocrine or paracrine stimulation (Ekstrand et al., 1991; Guha et al., 1995; Hermanson et al., 1992; Nistér et al., 1988; Sara et al., 1986; Trojan et al., 1992; Wong et al., 1987). The receptor overexpression is sometimes due to amplification of the receptor gene (Ekstrand et al., 1991; Fleming et al., 1992). Furthermore, a subset of GBMs harbors an activating deletion of the EGFR (the most common being the EGFRvIII with an in frame deletion of 600 amino acids in the extracellular domain), generating a receptor that signals in the absence of ligand binding (Ekstrand et al., 1994). The tumor suppressor gene PTEN is also commonly mutated or deleted in GBMs (Li et al., 1997; Wang et al., 1997), leading to greatly facilitated signaling through the PI3K/AKT pathway. Activating RAS mutations are seen in a variety of human tumors but are not common in gliomas (Bos, 1989), perhaps because overactive growth factor receptor signaling makes this unnecessary.

Loss of G₁ restriction point regulation

About 60% of GBMs show INK4a-ARF deletion or inactivation (Costello et al., 1996; Ichimura et al., 1996) resulting in deregulation of both the RB and p53 pathways. The remaining tumors often have p53 mutation (30-40 %) and loss of the remaining p53 allele as noted by loss of heterozygosity (LOH) of chromosome 17 (Louis, 1994), in combination with either RB deletion or amplification of CDK4 (Ichimura et al., 2000; Ichimura et al.,
1996), thereby affecting both pathways as well. If neither p14ARF nor p53 is directly affected, p53 may still be inactivated by MDM2 amplification and overexpression (Reifenberger et al., 1993).

**Primary and secondary glioblastomas**

Although histologically indistinguishable, GBM may clinically be divided into two entities. “Primary” GBMs present with no prior history, de novo, as highly malignant grade IV tumors, whereas “secondary” GBMs progress from gliomas of lower grades over a number of years. Primary GBM generally strike an older population with a peak between 45-70 years, while secondary GBM affect younger people with a peak between 25-50 years. While some of the de novo tumors may develop from clinically silent tumors, analysis of genetic aberrations indicates that the majority of tumors do not, and that there exist a number of molecular paths by which GBMs may develop (Figure 2).

Characteristic for primary GBM is deletions of the INK4a-ARF and INK4b gene on chromosome 9p which occur in about 40% (Ichimura et al., 1996; Schmidt et al., 1994). In an additional 14-20% the INK4a-ARF gene is silenced by methylation of the 5′ CpG islands of the gene (Gonzalez-Zulueta et al., 1995; Merlo et al., 1995). EGFR amplification and overexpression is also much more frequent in primary GBM, with amplification being present in about 30-40%, while very rarely seen in secondary GBM (Tohma et al., 1998). EGFR expression measured by immunostaining in the same study showed EGFR staining in >60% of primary GBM compared to <10% in secondary GBM.

Chromosome 10 harbors the PTEN gene on its long arm. While loss of heterozygosity on chromosome 10 occurs in both primary GBM and as anaplastic astrocytomas progress to secondary GBM, primary GBMs have a much higher frequency of PTEN mutations (32% vs 4%) (Tohma et al., 1998). The LOH seen in secondary GBM more often affect regions of 10q, distal to the PTEN locus, whereas primary GBM tend to have the whole chromosome deleted (Fujisawa et al., 1999).

Secondary GBM are defined by their previous history as lower grade gliomas. Almost 60% of diffuse grade II astrocytomas have LOH of chromosome 17p, though a majority of cases are actually not identifiable cytogenetically due to recombination and reduplication (el-Azouzi et al., 1989; James et al., 1989). The TP53 gene is located on chromosome 17p and is the target of the allelic loss. In a majority of cases this is associated with
mutations of the remaining TP53 gene (Ohgaki et al., 1993; Rasheed et al., 1994; von Deimling et al., 1992a). The frequency of TP53 inactivation does not increase with malignancy indicating that this is an early event in gliomagenesis. Over 90% of TP53 mutations seen in high grade secondary GBMs were already present in earlier biopsies from lower grade tumors (Watanabe et al., 1997; Watanabe et al., 1996).

Deletions of the distal end of 10p has been identified in grade II astrocytomas but no genes have been implicated in this region (Ichimura et al., 1998). LOH of chromosome 13q including the RB gene is seen in about 25% of both astrocytoma grade II and III, though tumors seem to retain the remaining RB allele (Ichimura et al., 1996).

Overexpression of PDGFRα and presence of the different PDGF isoforms is also an early event being present in a majority of gliomas of all grades, including grade II astrocytomas, also indicating that an autocrine loop of PDGF stimulation may have a role in gliomagenesis (Guha et al., 1995; Hermanson et al., 1992; Lokker et al., 2002). Both primary and secondary GBM overexpress PDGF and PDGFR, but the role of PDGF in the development of secondary GBMs is probably more important as EGFR expression and amplification is not seen in this group or in the preceding lower grade tumors.

When All tumors evolve to AA, several additional genetic aberrations are seen. About 10% have homozygous deletions of INK4a-ARF, usually seen in primary GBMs. Amplification of genes is very rare in grade II astrocytomas but begin to appear in anaplastic astocytomas. CDK4 is amplified in approximately 8% of AA.

**Figure 2. Different developmental paths for primary and secondary GBM.**
Using mouse models to investigate gliomas

Several strategies have been employed to model gliomas in rodents, but the two main objectives have always been to identify causative genetic changes in tumors, thereby identifying possible new targets for treatments and broadening the general understanding of brain tumor biology, and to create a representative system in which to test potential therapies. The first models to be developed were either based on transplantation of cultured human or rodent brain tumor cells into, often immunodeficient, recipient animals (Kobayashi et al., 1980; Shapiro et al., 1979), or induction of de novo brain tumors in rodents with mutagens, such as nitrosourea (Kleihues et al., 1976; Koestner et al., 1971). Both models have strengths and drawbacks. The xeno- or allograft models are highly reproducible with respect to tumor take rate, growth rate, and survival, but they can never quite recapitulate the infiltrative characteristics of especially glial tumors and remain a “metastatic” model (Finkelstein et al., 1994). The host animals’ immune response is often not taken into account, as immunodeficient mice are usually used in order to accommodate human glioma cells. In addition, a strong selective pressure is seen in all forms of cell culturing and the cells used for transplantation experiments may not well represent the original tumor. Tumors induced by mutagenic alkylating agents, on the other hand, do grow intrinsically within the brain tissue and histologically resemble gliomas (Koestner et al., 1971), but, as the lesions are caused by unknown mutations, the genetic profile is unknown and differs between tumors, making them non-reproducible.

As the group of genetic aberrations identified in human brain tumors has increased, the question has arisen as to which are involved in tumor initiation and progression and which are “innocent bystanders”, perhaps caused by previous mutations. The only way to determine such causality is to recreate these mutations in animal models. It is important to remember that although intracellular pathways and cell cycle components are preserved between mouse and man, mouse cells are more easily transformed. In response to challenge with activated RAS, mouse cells can be transformed if either \( p53 \) or \( \text{Ink4a-Arf} \) is lost, while human cells need the loss of both loci in order to be transformed and not enter senescence or apoptosis (Serrano et al., 1997).

Parallel to the advent of molecular diagnostics, the methods to generate mice with gain-of-function (transgenic mice; Figure 3A) and loss-of-function (targeted deletions; Figure 3B) alterations have been developed. The central idea of genetic mouse models of gliomas have been recreate the genotype seen in the human tumors in cells of mice to determine if this will
Figure 3. Germline genetic modification of mice. A. Transgenic mouse. DNA constructs with a tissue specific promoter preceding the (onco-) gene to be expressed are injected into a fertilized oocyte. The sequence is incorporated into the genome and is present in every cell in the resulting mouse but only expressed in cells where the promoter is activated. B. Targeted deletion (inactivation) of genes in mice. The gene to be inactivated is manipulated in vitro. A neomycin resistance gene (neoR) is inserted, and replaces a gene sequence essential for protein function. This modified genomic sequence is transfected into embryonic stem (ES) cells derived from a blastocyst and incorporated into the genome by homologous recombination. Addition of normally cytotoxic neomycin will allow for selection of cells where the DNA has been incorporated. ES cell clones are tested for correct recombination (not shown) and injected into blastocysts, which in turn are reimplanted into a pseudopregnant mouse. If the ES cells contribute to the germline, breeding of chimeric mice can result in a mouse line completely derived from the ES cell line. ES cells containing the modified construct are taken from mice with a coat color that differs from the donor of the reimplanted blastocyst.
result in tumors, and if these tumors have the corresponding phenotype. Transgenic mice often have expression of an oncogene driven by a tissue-specific promoter, for models of glioma this would be a promoter of a protein expressed in glial cells or glial precursor cells such as glial fibrillary acidic protein (GFAP) or nestin (Aguzzi et al., 1995). Originally, targeted deletions were achieved by germline deletion of a given gene in every cell in the mouse. However, recent methods of conditional gene deletions have been developed, in which the tumor suppressor can be turned off in a tissue- and/or temporal-specific manner, making studies of otherwise fatal genetic defects possible (Macleod and Jacks, 1999). By crossing modified strains of mice, the cooperative effects of specific genetic alterations can be identified. Tumors in these mice often arise due to secondary mutations, made possible by the original alteration(s), and the models are therefore well suited for identifying novel factors involved in initiation or progression of tumors. These models can produce very accurate tumors but, as in the case of all modeling systems, there are some negative aspects. Apart from being costly, the breeding and genotyping required can be extensive, especially if a combination of more than two genetic mutations is sought.

A second method for genetic alteration is by somatic cell gene transfer using retroviral vectors to the neonatal mouse brain. Two systems have been developed. One uses replication competent Moloney murine leukemia virus (MMLV) as delivery vector of specific genes and is the one used to induce tumors in papers I-III (discussed in Present investigation below). Injection of viral vectors into neonatal animals will allow non-specific gene-transfer by viral infection of proliferating cells (Uhrbom et al., 1998). The other system employs an ALV (avian leukemia virus)-based replication competent system (Figure 4). ALV-based RCAS vectors containing a gene, or combination of genes, are transferred/injected into the brain of transgenic mice expressing the RCAS receptor, tv-a, on target cells (Holland et al., 1998a; Holland et al., 1998b). Mouse lines expressing the tv-a receptor driven by the astrocyte specific GFAP promoter or the nestin promoter (expressed in CNS progenitor cells) have been generated. In this system relatively few cells are infected which reduces the chances for secondary mutations, setting it apart from the MMLV-model and the systems using germ-line mutations. The RCAS/tv-a system is therefore useful for studying if the effects of single or multiple defined genetic changes suffice to result in tumor formation. However, this method is not effective to detect additional alterations that contribute to tumorigenesis. The unknown factors of the systems are the effects of the retroviral injection itself. Local inflammation or glial scar formation could theoretically affect tumor formation.
Figure 4. The RCAS/tv-a system of somatic gene transfer. The (onco-) gene to be transferred is inserted into the viral genome and the modified RCAS virus is propagated in avian cells in vitro. One µl of pelleted virus-producing cells is injected into the brain of transgenic mice expressing the RCAS receptor, tv-a, under the control of a tissue specific promoter. Only cells expressing the receptor can be infected allowing introduction of a foreign gene into specific cell types or tissues. The gene product is expressed in the infected cells, but the virus does not replicate in mammalian cells. (*-constitutively activated protein)

Astrocytoma models

Diffuse astrocytomas (grade II and III) have been successfully modeled using several different approaches. By manipulating signal transduction pathways or using cell specific inactivation of cell cycle arrest pathways, GFAP-expressing cells can develop into astrocytomas of different grades. When driven by a GFAP promoter in transgenic mice, constitutively active Ras induced astrocytomas (WHO grade II-III) at high frequencies (Ding et al., 2001). Two transgenic mouse lines with different levels of Ras expression were created and it appears that the characteristics of the resultant astrocytomas depend on Ras-gene expression dosage.

Overactivation of signal transduction pathways is also used in a transgenic model with the intracellular tyrosine kinase v-src, the viral oncogene of Rous Sarcoma Virus (RSV). The rationale to use v-src was based on earlier experiments with dogs that develop gliomas after intracerebral injection of RSV (Bigner et al., 1969; Rabotti et al., 1966), and studies that showed src to interact with the same signaling pathways as the EGFR and PDGFR (Haas et al., 2000; Luttrell et al., 1994; McGlade et al., 1992). Transgenic mice with v-src co-expressed with GFAP in astrocytes...
developed astrocytoma-like tumors of varying malignancies at a frequency of 14.4% (Weissenberger et al., 1997).

As the cell cycle regulator RB is inactivated by INK4A-ARF deletion, CDK4 overexpression, or direct RB mutations in 70-80% of all high-grade gliomas (Henson et al., 1994; Ueki et al., 1996), a transgenic astrocytoma model was developed by tissue specific inactivation of the Rb family proteins. A mutant of the oncovirus SV40 T antigen, T_{121}, which binds to and inactivates Rb, p107, and p130 (the other members of the Rb family), was expressed in mice under the GFAP promoter. This lead to brain abnormalities and early post-natal deaths, but when the system was modified so that T_{121} was turned on later in development, mice developed lethal anaplastic astrocytomas in close to 100% of cases within 10 months (Xiao et al., 2002). Proteins of the RB family seem to have redundant functions in mice as conditional Rb deletion alone in GFAP expressing cells had no effect on astrocyte maturation and did not cause tumors (Marino et al., 2000).

**Oligodendroglioma models**
The name oligodendroglioma implies a histogenesis from the oligodendroglial lineage and is based on morphological similarities of the tumor cells and oligodendrocytes. Oligodendrogliomas, unlike astrocytomas, do not express GFAP and are therefore thought to have a different cell-of-origin. Several lines of evidence implicate overactive signaling in the genesis of these tumors. PDGF-A and -B are co-expressed with both PDGFRs (-α and-β) in almost all oligodendrogliomas (Di Rocco et al., 1998) and in about one half of grade II-III oligodendrogliomas overexpression of the EGFR is also seen (Reifenberger et al., 1996). As mentioned above, there is evidence that the OPC can be kept in an undifferentiated, proliferating stage by continuous PDGF stimulation (Noble et al., 1988; Raff et al., 1983). Unpublished studies by Deinhardt have shown that the Simian Sarcoma Virus (SSV), viral homologue of c-sis/PDGFB, could induce malignant brain tumors when injected into the brains of newborn marmosets. Many of the resulting tumors resembled anaplastic oligodendrogliomas or GBM with large oligodendroglioma components (Nistér and Westermark, 1998).

With these facts in mind, two mouse model systems involving the somatic gene transfer of PDGFB to brain cells have been used to create oligodendrogliomas or oligodendroglioma-like lesions of different grades. The model to first demonstrate this is used in papers I-III, and utilized the replication competent MMLV vector to transfer the PDGFB gene to proliferating cells by injection into the neonatal mouse brain (Uhrbom et al.,
1998). These mice developed brain tumors at a frequency of about 40-60%. The tumors had similarities with human GBMs or PNETs, but some did have characteristics of oligodendrogliomas grade II and III.

Another model for oligodendroglioma uses the RCAS/tv-a system of gene transfer. By using transgenic mice with the tv-a receptor in either nestin or GFAP expressing cell, the PDGFB gene could be transferred to defined cell populations in the neonatal mouse brain (Dai et al., 2001). PDGF-B gene transfer to neural progenitors induced oligodendrogliomas, predominantly low grade, at a frequency of about 60%. If the gene instead was transferred to more mature cells expressing GFAP, about 40% of mice developed either oligodendrogliomas or mixed oligoastrocytomas. By crossbreeding these tv-a transgenic mice with a second mouse strain, lacking the tumor suppressor gene locus Ink4a-Arf, combinatory effects were obtained. Loss of cell cycle arrest pathways by Ink4a-Arf deletion increased the proportion of anaplastic tumors, shortened latency in nestin tv-a mice, and increased total number of tumors as well as malignancy grade in GFAP tv-a mice.

Mice with Ink4a-Arf loss develop fibrosarcomas or lymphoid malignancies within 4-8 months, but have not been reported to develop brain tumors (Serrano et al., 1996). Surprisingly, approximately 10% of mice deficient only for Arf (retaining functional Ink4a) develop oligodendrogliomas (Kamijo et al., 1999). The effect may be mediated by inactivation of p53, but this is unclear. Mice with p53 loss have not been reported to develop brain tumors spontaneously (Donehower et al., 1992), not even if p53 is conditionally inactivated in only GFAP expressing cells (Marino et al., 2000). Since oligodendrogliomas lack GFAP, spontaneously occurring tumors in Arf null mice may develop from an earlier precursor.

Further evidence of the important role of overactive signaling in oligodendroglioma development comes from reports that transgenic mice expressing the oncogene v-erbB (the constitutively active homolog of EGFR) in astrocytes and glial precursors using S-100β promoter developed oligodendrogliomas (Weiss et al., 2003). Tumor grade and penetrance were increased if the p53 pathway was inactivated. This was seen when crossing transgenic v-erbB/S-100β mice with p53 or Ink4a-Arf heterozygotes or Ink4a-Arf null mice, but not when crossbreeding with Rb1 heterozygotes. As for several other tumor models, it is clear that many different genetic defects can lead to the same end result, presumably due to similarities in the resultant downstream signaling pathways.
Glioblastoma multiforme models

The heterogeneous histology of GBM and the fact that the human criteria for GBM may not be optimal for evaluating mouse tumors can make histopathological diagnosis of such mouse tumors difficult. The histological hallmarks of GBM are infiltrative growth, nuclear polymorphism, nuclear atypia, active mitotic activity, microvascular proliferation, and necrosis (Kleihues et al., 2002). Using these criteria, a few of the previous models of both astrocytomas and oligodendrogliomas can in their most malignant form be called GBM. A few late tumors in v-src/GFAP transgenic mice and a majority of tumors in the MMLV/PDGFB model exhibited most of the features mentioned above. The first model to unequivocally produce GBM used the RCAS/tv-a system to transfer mutant, constitutively active forms of K-Ras and Akt to nestin expressing neuroglial progenitor cells (Holland et al., 2000). Ras and Akt are members of two separate, but interacting, signaling pathways downstream of several growth factor receptors, and both are activated in a majority of human GBM (Guha et al., 1997; Holland et al., 2000). The expression of activated Akt could be considered a combination of PI3K mediated growth factor signaling and PTEN deletion, as the inhibitory function of PTEN lies just upstream of AKT. Activation of only one pathway did not lead to tumor formation, nor could the combination of Ras and Akt induce tumors in GFAP expressing astrocytes. This demonstrates the importance of both the activation of certain signaling cascades and the differentiation status of the cell of origin. The reason the earlier described transgenic Ras model could produce astrocytomas in GFAP expressing cells without other engineered mutations could be related to higher levels of Ras expression and the fact that all GFAP-expressing cells in the brain are affected, increasing the probability that one of these cells acquires additional contributory mutations required for tumor formation. Another possibility is that the transgene is expressed in earlier, more immature and responsive, GFAP positive cells in the prenatal mouse brain.

Another recent model initiates GBM by loss of tumor suppressors, rather than oncogene overexpression. By breeding mice with heterozygous for p53 with mice heterozygous for Nf1 (the mouse equivalent of human NF1, the gene), cis-heterozygotes could be generated. These mice have deletions of one of the alleles of both genes adjacent to each other on the same chromosome. Loss of the other chromosome results in loss of the remaining alleles of both Nf1 and p53, generating cells that are double null for Nf1 and p53. This mouse line develops astrocytomas and GBM of varying frequency, and PCR analysis demonstrates loss of the remaining alleles of these genes in a majority of tumors (Reilly et al., 2000). Older mice tended to have high-grade lesions and this model could simulate the spectrum and maybe the
progression seen in secondary GBM. Despite apparent differences, these two GBM models have similarities. Since neurofibromin functions as an inhibitor of Ras activity, the \( Nf1 \) deletion will therefore result in deregulated, overactive Ras signaling. Apparently not sufficient to single-handedly form tumors, \( Nf1 \) deletion needed the cooperative effect of \( p53 \) loss and the resulting deregulation of cell cycle control.

In summary, the brain tumor models discussed above manipulate either growth factor receptor signaling pathways, cell cycle arrest pathways, or both, and effects have been shown to depend on the cell-of-origin. Regardless of method, valuable information regarding the role of known common genetic alterations in tumorigenesis or tumor progression has been, and may yet be, gained.

**Table 1. Glioma models in mice**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Tumor Suppressor Gene</th>
<th>Oncogene (over-expressed or activated)</th>
<th>Cell of origin/ cell affected</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AII</td>
<td>( Nf1+/) and ( p53+/) in cis</td>
<td>V12Ha-Ras</td>
<td>astrocyte (GFAP+)</td>
<td>Ding</td>
</tr>
<tr>
<td>OII</td>
<td>Arf/-</td>
<td>PDGFB</td>
<td>progenitor (nestin-)</td>
<td>Dai</td>
</tr>
<tr>
<td>OII</td>
<td>( Nf1+/) and ( p53+/) in cis</td>
<td>v-src</td>
<td>astrocyte (GFAP+)</td>
<td>Reilly</td>
</tr>
<tr>
<td>OII</td>
<td>Ink4a-Arf+/+ or Ink4a-Arf/-</td>
<td>PDGFB</td>
<td>astrocyte (GFAP+)</td>
<td>Dai</td>
</tr>
<tr>
<td>AA</td>
<td>V12Ha-Ras</td>
<td>PDGFB</td>
<td>astrocyte (GFAP+)</td>
<td>Dai</td>
</tr>
<tr>
<td>(WHO III)</td>
<td>( Nf1+/) and ( p53+/) in cis</td>
<td>v-src</td>
<td>astrocyte (GFAP+)</td>
<td>Reilly</td>
</tr>
<tr>
<td>GBM</td>
<td>Ink4a-Arf/-</td>
<td>PDGFB</td>
<td>progenitor (nestin+)</td>
<td>Dai</td>
</tr>
<tr>
<td>(WHO IV)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AII - diffuse low grade astrocytoma (WHO II), OII - oligodendroglioma, OAIIOII-oligo-astrocytoma, AA- anaplastic astrocytoma, and GBM- glioblastoma multiforme.
Pros and cons of genetic glioma mouse models

Different questions can be addressed depending on model used. Somatic cell gene transfer models may be more efficient than germline mutations if a combination of more than two mutations is to be studied. The MMLV model of gene transfer used in this thesis can help identify unknown genes cooperating in tumorigenesis by studying the genome surrounding viral insertion sites. The models also vary in their ability to reproduce infiltration of normal brain tissue, another hallmark of malignant brain tumors. Several of the models employing germline modifications have difficulties mimicking this characteristic as “normal tissue”, although not overtly transformed, still harbors genetic alterations and may respond to invasion in a different manner. Sometimes it may be difficult to distinguish increased “normal” cell proliferation from diffuse low grade tumor. If only the tumor cells have been affected by the initiating stimuli, there will be an expansion of transformed tumor cells, which may grow infiltrative but readily be discerned from normal tissue.

The obvious difficulty with most genetically based models regarding drug studies is the stochastic nature of tumor development. There will be a need for in vivo assessment of mice at risk to determine when tumors are developing and treatment should commence. Such systems for screening mice with MRI are being developed (Koutcher et al., 2002).

Targeting receptor signaling in gliomas

The importance of receptor activation and hyperactive signaling through the RAS/MAPK and PI3K/AKT pathways is underscored by the mouse models described above. Most models of GBM seem to require some degree of increased signaling through these pathways. Returning to human gliomas with their overexpression of several growth factor receptors, it is easy to see the rationale for the development of specific inhibitors of receptor activation or of activation of effector molecules further downstream in the signaling cascade. The main advantage of targeting receptor activity is the relative specific overexpression of the EGFR and PDGFR in GBMs. Imatinib mesylate (Glivec; Gleevec; STI571; CGP57148B) is a tyrosine kinase inhibitor of PDGFRα and-β, c-Abl, and c-Kit at nano- to micromolar concentrations. It has comparably low activity against EGFR, VEGFR and insulin receptor kinases (Buchdunger et al., 2000; Buchdunger et al., 1996; Buchdunger et al., 1995). As a small 2-phenylaminopyrimidine derivative, it can pass over the cell membrane to reversibly block the ATP binding pocket in the kinase domain. It is used in clinical practice against chronic myelocytic leukemia (CML), a malignancy driven by a rearranged and
constitutively active c-Abl (Capdeville et al., 2002), and is in several clinical studies for glioma treatment. Gifitinib (ZD1839; Iressa) is a selective EGFR inhibitor with relatively benign adverse effects and is the one to have reached furthest in clinical trials (Ciardiello and Tortora, 2001; Wakeling et al., 1996). Though there are varying results from therapeutic studies of other tumor types it is now in several clinical trials for GBM. It has been shown to have effect on intracranially xenografted EGFR expressing tumor cells in a mouse transplant model. Interestingly, it had little effect on cells expressing the mutated EGFRvIII (Heimberger et al., 2002).

Several points of possible inhibition in the intracellular cascades may not be suitable because of their essential functions in all cells normal metabolic activity. Insulin signaling, for example, is mediated to a large extent through the PI3K/AKT pathway (Cross et al., 1995; Hadari et al., 1992) and non-selective block of this pathway is toxic in vivo (Stein and Waterfield, 2000). Yet, it should be possible to find points of intervention in the intracellular pathways that are characteristic and specific for gliomas. It seems that the different isoforms of PI3K and AKT have different roles with varying specificities for signaling in metabolic pathways or oncogenic/survival pathways (Chen et al., 2001; Cho et al., 2001).

The mTOR protein is active downstream of AKT and its activity results in p70/S6K mediated cell growth through inactivation of the translational repressor 4E-BP1. An inhibitor of mTOR, CCI-779 is now in clinical trials for GBM treatment. Both in vitro and in vivo experiments have shown the inhibitor to have effect on cell lines and xenografts with activation of the PI3K/AKT pathway, where activation could result either from oncogenic signaling or loss of PTEN (Geoerger et al., 2001; Neshat et al., 2001). As mentioned above, the tyrosine kinase receptors present in gliomas share to a large extent signaling pathways and blocking one receptor may not be sufficient. Chakravati et al. showed that a glioma cell line’s insensitivity to an EGFR inhibitor, in spite of EGFR expression and activation, could be explained by IGFR signaling through the PI3K/AKT pathway (Chakravarti et al., 2002).

In conclusion, therapeutic strategies aimed at blocking signaling downstream of growth factor receptors are the beginning of a new type of rational cancer treatment, aimed at specific, sometimes causal mechanisms of cancer. It is still unclear if solitary therapy will suffice, or if more complex approaches with combinations of inhibitors or other forms of chemotherapy will be needed. Additional knowledge of the complex
network of intracellular signaling and cell cycle control is necessary before the goal of individually tailored treatments can be a reality.

Table 2. A selection of inhibitors of tyrosine kinase receptors and an mTOR inhibitor in GBM clinical trials

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Point of inhibition</th>
<th>Manufacturer</th>
<th>Clinical trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>STI-571 (Glivec)</td>
<td>PDGFR, bcr-Abl, c-kit</td>
<td>Novartis</td>
<td>phase I-II</td>
</tr>
<tr>
<td>ZD 1839 (Iressa)</td>
<td>EGFR</td>
<td>Astra Zeneca</td>
<td>phase I-II</td>
</tr>
<tr>
<td>OSI-774</td>
<td>EGFR</td>
<td>Oncogene Sciences</td>
<td>phase I-II</td>
</tr>
<tr>
<td>R115777</td>
<td>RAS</td>
<td>Janssen</td>
<td>phase I-II</td>
</tr>
<tr>
<td>CCI-779</td>
<td>mTOR (downstream of AKT)</td>
<td>Wyeth-Ayerst</td>
<td>phase II</td>
</tr>
</tbody>
</table>
Present investigation

Mouse model
The mouse model used in papers I-III is based on overexpression of PDGF-B with a retroviral vector in neonatal mouse brain. PDGF-B coding and control constructs were made in parallel. The constructs were co-transfected with a neomycin resistance plasmid into NIH 3T3 cells. After selection with G418 (neomycin equivalent), the clones were tested with Northern blotting for viral expression. Clones with high expression of PDGFB and control virus were selected and virus rescued by superinfection of helper virus, a replication competent Moloney murine leukemia virus (MMLV) and stored in aliquots at –70°C. Virus titer was assessed by a focus forming assay using NIH 3T3 cells.

Within the first 24 hours of birth, mice were injected with 10 µl of viral supernatant, containing 2x10^3 focus forming units. Viral solutions were slowly thawed on ice prior to injection. Injection was performed free-handedly with a 50 µl Hamilton syringe using a needle 0.4 mm wide, placed 2 mm down into the right hemisphere.

If mice died within two weeks, they were not included in the study. Animals were then followed every to every other day for signs of disease. The most common early symptoms were lethargy and weight loss. Sometimes fast development of neurological signs such as ataxia could be seen, and in a few cases enlargement of the skull was apparent. If animals showed signs of disease they were sacrificed immediately. In paper 1, only the cerebrum was analyzed while in paper 3, the cerebellum and brain stem were included as well. If tumors were macroscopically visible, brains were sectioned through the tumor and one part (preferably posterior part) was taken for histopathological diagnosis and fixed in 4% formaldehyde and paraffin embedded before sectioning. In paper 1, the other (usually anterior) part was divided in two and frozen in –70°C for DNA preparation and analysis of viral integration. In paper II, macroscopically visible tumors
were dissected out, mechanically dissociated and plated for cell culture. In paper III, tumor material was if possible also taken for cell culture, and carefully dissected and frozen for analysis of DNA content with flow cytometry.

Figure 5. The PDGFB/MMLV mouse model for brain tumors
Paper I. Induction of brain tumors in mice using a recombinant platelet-derived growth factor B-chain retrovirus

This study established the model also used in papers II and III. Several lines of evidence implicate hyperactive PDGF stimulation in the genesis of brain tumors. The first comes from experiments by Deinhardt during the late 1970’s using SSV (viral homologue of c-sis/PDGFB) to induce tumors. When injected into the brains of marmoset neonates, these animals subsequently developed highly malignant brain tumors resembling both GBM, PNET and sometimes anaplastic oligodendrogliomas (Deinhardt, 1980; Nistér and Westermark, 1998). Further circumstantial evidence comes from studies of glial progenitor cells, which have been suggested to be the cells-of-origin in some gliomas. GBMs are heterogeneous tumors in several ways, having both immature and differentiated areas, and may have areas of astrocytic appearance side by side with areas of oligodendroglioma histology. This implies that the cell-of-origin must be either a cell that can dedifferentiate, or a glial progenitor cell with multi-, oligo-, or bipotential developmental capabilities. The OPC expresses the PDGFRα and can be kept in a proliferating, undifferentiated stage by continuous PDGF stimulation in vitro. Thirdly, one characteristic of AII, AA and secondary GBMs is the overexpression of PDGFRα together with expression of the PDGF isoforms. The fact that the potential autocrine stimulation is present in all grades also indicates that it is an early event and may have a role in tumor initiation.

The aim of this study was to determine if overexpression of PDGF-B with a retrovirus in the brain of neonatal mice could induce the formation of gliomas. The main PDGFRα expressing cell in the newborn mouse brain is the OPC (Ellison and de Vellis, 1994; Pringle et al., 1992; Yeh et al., 1993), and would be the cell with most potential to respond to the PDGF-B stimulation.

We show that PDGF-B overexpression in neonatal mouse brain by retroviral transfer can induce brain tumors in 40% of wild type C57Bl6 mice. Tumors developed within 14-29 weeks and no additional tumors were seen during the remaining 36 weeks of the study. No tumors developed in mice injected with control virus and helper virus, indicating that the tumorigenic potential of the system relies on the PDGF-B overexpression, with or
without spontaneous mutations or mutagenic results of the retroviral integration.

The tumors had certain histological hallmarks of GBM, such as pseudopallisading cells around necrotic foci, areas with hemorrhages, aberrant vessels, and diffuse infiltrative growth. Cells were usually small and polygonal with an immature appearance and mitoses were seen. A pattern of ribbon-like secondary structure, sometimes seen in human primitive neuroectodermal tumors (PNETs)/medulloblastomas and oligodendrogliomas, was present in several tumors in the mouse system. All tumors had the retroviral \textit{PDGFB} sequence incorporated into the DNA, as shown by Southern blot analysis, and we could show by \textit{in situ} hybridization that all tumors, but not surrounding normal brain tissue, co-expressed PDGF-B and PDGFR\textsubscript{α} mRNA. PDGFR\textsubscript{β} mRNA was not seen in the tumors. Progenitor cells of the nervous system characteristically express nestin while GFAP expression is mainly seen in mature astrocytes. Immunohistochemistry showed the tumor cells to express nestin, while GFAP could be detected in surrounding reactive astrocytes, but not in actual tumor cells. The tumors also stained weakly for neurofilament, normally found in cells of the neural lineage. These findings are in line with the histological picture of immature tumors.

The transforming effect of PDGF autocrine stimulation has been seen in several studies. Pech et al. could induce fibrosacomas in mice by subcutaneous injection of a PDGF-B coding retrovirus, though in their study, intracranial injection did not result in tumor formation. The fibrosacromas were generally polyclonal with clonal subpopulations (Pech et al., 1989). \textit{In vitro} studies showed that the transformed cell type caused by infection with simian sarcoma virus (SSV/\textit{v-sis}) could be reverted by blocking the receptors with suramin (Betsholtz et al., 1986a) or PDGF antibodies (Johnsson et al., 1985). Equivalent studies were later carried out on a glioma cell line with an autocrine PDGF loop. The phenotype of the established tumor cell line could be reverted by the same means, indicating that also malignant tumor cells may still depend on PDGF activation (Vassbotn et al., 1994).

With this in mind, what is the mechanism for tumorigenesis in the MMLV/PDGFB system? As noted above, three options are possible. Either PDGFB overexpression alone causes the transformation, or it does so in combination with spontaneous mutations or mutations induced by retroviral integration. Although many different cells may be infected by the virus, cells with PDGFR\textsubscript{α}, such as neuroglial progenitor cells, will have an \textit{à priori} growth advantage in the system. The normal response in adult cells to very
strong mitogenic signaling is to go into senescence or apoptosis (Serrano et al., 1997). One would expect some additional change leading to loss of G₁ control to occur in order for the PDGF stimulation to be allowed to continue. There may be some form of G₁-control inactivation by the retrovirus itself or retroviral mutagenesis may cause loss of control by transcriptional activation of oncogenes, or inactivation of tumor suppressor genes. Unpublished observations by our group have shown that if PDGF-B was expressed in transgenic mice under the GFAP promotor, tumors only developed in mice lacking wild type p53. The discrepancy between the two systems may in most respects be due to the use of completely different methods. Studies with the RCAS/tv-a system also showed that nestin expressing cells were more readily transformed than GFAP expressing cells (Dai et al., 2001; Holland et al., 2000; Holland et al., 1998a). It seems that progenitor cells are more permissive to excessive mitogenic stimuli and transforming oncogenes. However, it has been shown that loss of the Ink4a-Arf locus enabled astrocytes to dedifferentiate in response to EGFR activation (Bachoo et al., 2002). Ink4a-Arf loss also allowed the combination of RAS and AKT in the RCAS/tv-a system to form tumors in GFAP expressing cells (Uhrbom et al., 2002). This indicates that deregulation of G₁ control and progenitor status may be two sides of the same coin.

CNS progenitor cells, which have the ability to develop into both motor neurons and oligodendrocytes, have recently been identified. These cells express the Olig1 and Olig2 genes (coding for transcription factors) (Lu et al., 2002; Lu et al., 2000; Zhou et al., 2000). With this backdrop, the histological picture of MMLV/PDGFB-induced tumors may more easily be understood. The immature tumors with some characteristics of GBM and malignant oligodendrogliomas, as well as PNET like morphology (and weak expression of neurofilament) could stem from the same type of progenitor cell(s).

In addition to differentiation status of the cell-of-origin, there may be a question of oncogenic “dose”. Tumor development varied according to level of activated Ras expression in the Ras tumor model (Ding et al., 2001). It could be that viral PDGF-B dose is much higher in the MMLV/PDGFB system than in the PDGF-B/GFAP transgenic mice.

If tumors do arise as a result of the combinatory effect of viral mutagenesis and PDGF-B autocrine stimulation, this system can be used to identify cooperating gene mutations. The insertions are located using a tag in the retrovirus, and by identifying the viral insertion sites and surrounding DNA sequence, genes, or promoters/repressors of genes, involved in the
tumorigenic process may be identified. Analysis of tumor DNA from this series of mouse tumors has revealed three integrations in the \(p53\) locus. Since the \(p53\) protein functions as a tetramer complex, a mutation in a single allele may inactivate almost all \(p53\) function.

**Paper II. Role of initiating stimuli on growth of PDGF-B-induced mouse brain tumor cells**

Once set up, the brain tumor model generated additional questions. Were the tumor cells still dependent on the initiating stimuli, i.e. could the cells be inhibited by blocking activation of the PDGFR? As described above, in vitro studies of SSV transformed cells and glioma cells showed that the malignant phenotype can be reverted by blocking PDGF (Betsholtz et al., 1986a; Johnsson et al., 1985; Vassbotn et al., 1994). In a lymphoma model system in mice using conditional expression of the protooncogene \(MYC\) in hematopoietic cells, inactivation of the \(MYC\) transgene resulted in sustained remission in 80% of mice with manifest disease (Felsher and Bishop, 1999).

In order to study the role of continuous PDGF-B stimulation in our system, cells from tumors induced in Ink4a-Arf deficient and wild type mice were cultured in vitro. Tumors in paper I were nestin positive, indicating an immature origin or development. In order to retain the immature phenotype in vitro, cells were cultured using a protocol for neuronal stem cells. A defined medium (N2), with daily addition of bFGF, was used and cells were plated in culture dishes coated with fibronectin (Johe et al., 1996). In this medium only Inka4a-Arf\(^{-}\) tumor cells survived more than two passages.

Several experiments were performed to verify that the cultured cells were actually tumor cells. Integration of the PDGF-B cDNA in genomic DNA of the cultured cells was shown by PCR, and both early and late passages produced viral titers of \(10^3\)-\(10^4\) ffu/ml. As in situ hybridization of the original tumor (also in paper I) could only detect \(PDGF\) mRNA in the tumor and not in surrounding tissue, this supported the cells as being tumor-derived. The cultured cells, like the original tumor, were nestin positive and expressed the chondroitin sulphate proteoglycan NG2, also expressed by OPC and cells of mesenchymal origin (Nishiyama et al., 1991; Stallcup and Beasley, 1987), indicating that tumor cells may stem from an early glial progenitor. Cultured tumor cells were also able to form subcutaneous tumors in nude mice. Subcutaneous xenografts of cells from passage 27 formed nestin positive sarcoma-like tumors after about 6 weeks, and when replated in tissue culture cells again expressed NG2 and nestin.
Primary tumors expressed mRNA for PDGF-B and PDGFRα, but not PDGF-A or PDGFRβ, as shown in paper I. In early passages the situation was the same in vitro, but later passages also expressed PDGFRβ mRNA and protein. In parallel to the appearance of PDGFRβ, cell morphology changed from a bipolar shape with round nuclei, to a larger cell type with flattened morphology and larger cytoplasm. This probably represents a selection or adaptation to in vitro culture conditions. As can be expected from the expression pattern of mRNA and proteins, the PDGFRs were phosphorylated.

The phosphorylation of both PDGFRs was possible to inhibit using the tyrosine kinase inhibitor imatinib mesylate (Glivec). Interestingly, SELEX, a high affinity oligonucleotide aptamer that binds to and inhibits the PDGF-B chain (Green et al., 1996), only managed marginal inhibition. This may be due to an inability to remove already bound PDGF from the receptors. The aptamer also does not pass over the cell membrane and it is possible that the ligand receptor complex already forms intracellularly. When performing proliferation assays in the presence or absence of the inhibitors, Glivec showed a similar dose-dependent inhibition of cell growth, as it did for the receptor phosphorylation inhibition. The PDGF-B aptamer barely inhibited cell growth. With higher passage the cultured cells exhibited a faster proliferation rate and increased PDGF-B independence. In vitro progression is most likely the cause of this.

Although this is an in vitro study on mouse tumor cells, it supports the idea that tumors may remain dependent on continuous growth factor receptor activation. The logical continuation of the study would be to take the inhibitor used here to treat human brain tumors with high PDGFRα phosphorylation.

Paper III. Complementary effects of PDGF autocrine stimulation and p53 or Ink4a-Arf deletion in a mouse glioma model

Loss of G1 control and apoptosis response by inactivation or deletion of the tumor suppressor genes TP53 or INK4a-ARF are among the most common genetic changes seen in GBM. Specifically TP53 mutation, together with LOH on chromosome 17p has been linked to low grade gliomas and secondary GBM. This subgroup has also been linked to PDGFRα
overexpression, and amplification in some cases. In the MMLV/PDGFB
tumor model, loss of these specific tumor suppressor genes is probably not
required for tumorigenesis, as seen by cell culture experiments. Yet, other
brain tumor model systems have shown complementary effects of combined
loss of cell cycle control and oncogenic stimuli (Dai et al., 2001; Reilly et
al., 2000; Uhrbom et al., 2002). The same is true in model systems for
hematopoietic malignancies (Schmitt et al., 1999).

The aim of this study was to evaluate differences in tumor histology,
latency, and frequency, if PDGF-B over-expression were combined with p53
or Ink4a-Arf deletion.

The MMLV/PDGFB retrovirus was injected into newborn mice with p53
or Ink4a-Arf null background, as well as into their wild type (wt)
counterpart. The two knockout strains were bred on a similar background but
not crossbred, so both types of wt mice were used to determine strain-
specific differences. Wt mice developed tumors at a frequency of 55-60%.
Tumor incidence was higher than in paper I and could be due to strain
differences, but is most likely a result of evaluation of lower brain stem and
cerebellum in this study. This was not done in the previous study. There was
no significant difference in tumor latency between the wild type groups, and
median ages at sacrifice in tumor bearing mice were 123 days and 132 days
in p53 and Ink4a-Arf wt mice respectively.

Compared with their wild type counterpart, both sets of null mice
developed tumors earlier and more frequent. Mean ages at sacrifice for
tumor bearing animals were 56 days and 73 days for p53 and Ink4a-Arf null
respectively. Log-rank test of the Kaplan-Meier survival plots showed a
statistically significant difference between the null groups with tumors
appearing slightly earlier in p53 null mice. This difference was quite small
when compared with the difference to wild type mice. We here show that
either p53 or Ink4a-Arf loss facilitates tumorigenesis in this system.

Dai et al. also used PDGF-B overexpression in the RCAS/tv-a system to
induce oligodendroglioma-like lesions (Dai et al., 2001). When PDGF-B
was transduced into cells with an active nestin promoter,
 oligodendroglomas were induced, and when astrocytes having an active
GFAP promoter were transduced, oligodendrogliomas or mixed
oligoastrocytomas developed. In Ink4a-Arf null mice, tumor incidence and
malignancy increased and latency was shortened, but surprisingly p53
deficiency provided no cooperative effect in GFAP tv-a transgenes. The
tumor incidence was actually lower in mice lacking the p53 tumor
A possible explanation for this could be differences in target cells. All tumors induced in the MMLV/PDGFB system, regardless of genetic background, were nestin positive and GFAP negative, indicating a progenitor type cell-of-origin, while the study by Dai et al. only showed lack of cooperative effect when PDGF-B was overexpressed in GFAP expressing cells. The possibility of proviral insertion mutagenesis causing additional mutations is higher in our study, as a replication competent virus is used. The role of p53 as a sensor of DNA damage may be more important in this scenario and loss of function would then have a more impact.

Tumor histology and immunoreactivity against nestin, GFAP and vimentin did not differ between null and wild type tumors, nor did they differ from earlier studies. Tumors were undifferentiated and aggressive with high mitotic frequency, aberrant vessels, and areas of necrosis and hemorrhages. DNA content of frozen tumor tissue was also determined and all primary tumors analyzed were diploid. Although point mutations and balanced recombinations could not be excluded, gross chromosomal changes could. Several tumors were cultured in vitro and all except one Ink4a-Arf null cell culture remained diploid in early (3-6) passages. A few of both p53 and Ink4a-Arf null tumors turned aneuploid or tetraploid in later passages 12-15 (wild type cultures senesced before eight passages), but there was no difference between the two null groups.

Could the reason for the earlier appearance of tumors in null mice be due to signaling differences? Tumor material from only a few tumors of each genotype remained after the analyses above. Protein from two tumors of each of the null groups and four tumors of the wild type groups were separated on gels and analyzed. We found a tendency toward lower levels of PTEN and higher levels of AKT phosphorylation in the null tumors, though most prominent in the p53 null tumors, compared to wild type tumors and normal brain tissue from mice of similar ages. The phosphorylated/activated fraction of ERK 1 and 2 were relatively similar in all tumors, though higher than in normal tissue. A study has shown p53 to be a positive regulator of PTEN transcription and it seems that a large part of p53’s role in apoptosis and senescence is mediated through inhibition of AKT in this fashion (Stambolic et al., 2001). Conversely, PTEN, by blocking AKT’s activation of MDM2, prevents the latter’s inactivation of p53 (Mayo et al., 2002; Zhou et al., 2001). Recent studies show that PTEN also stabilizes p53 by phosphatase independent mechanisms, probably by direct binding to p53 (Freeman et al., 2003). Though not shown in gliomas directly, studies on breast cancer indicate that PTEN and p53 mutations are mutually exclusive (Kurose et al., 2002). PTEN mutation and deletion is also more common in
primary GBM (Fujisawa et al., 2000; Tohma et al., 1998). Loss of, or lack of up-regulation of p53 in response to oncogenic stimuli such as overactive PDGF-PDGFR signaling may be one reason for facilitated signaling through the PI3K/AKT pathway and perhaps a reason for faster tumor development. In the protein analysis of tumors and non-tumor brain tissue, normal \( p53^- \) and \( Ink4a-Arf^- \) tissue did not display reduced PTEN expression or increased AKT activation. The increased AKT activation may therefore be due to the combination of PDGF stimulation and loss of the tumor suppressors. There are most likely several other, perhaps more important factors at play here. Further studies should include protein analysis on a larger number of tumors. *In vitro* studies on PDGF-B stimulated primary cultures could also give valuable information.

Regardless of mechanism, the cooperative tumorigenic effects of PDGF-B stimulation and p53 loss is here demonstrated for the first time in an *in vivo* model, establishing a functional link between two common molecular changes in secondary GBM.

**Paper IV. Different glioblastoma multiforme subgroups exist with regards to EGFR and PDGFR\(\alpha\) activation**

In parallel to the mouse model work a series of human glioblastoma multiforme biopsies were collected and studied with regards to phosphorylation levels of two tyrosine kinase receptors known to be over-expressed in these tumors, the EGFR and the PDGFR\(\alpha\). The goal was to determine if and how receptor activation varied within the glioblastoma group, and if this was related to *EGFR* amplification or *TP53* mutation, common aberrations in primary and secondary GBMs respectively.

With the development of specific growth factor receptor inhibitors by research and pharmaceutical companies, a new potentially powerful treatment strategy against gliomas have emerged. Our study aimed at determining if there were different subgroups within primary GBM, with regard to functional dependence on either or both receptors.

In a first step, we have here semi-quantitatively assessed receptor phosphorylation and expression on the protein level in twenty-seven cases of human glioblastoma multiforme. In order to relate our findings to genetic subgroup we also examined tumors for \( p53 \) mutations and *EGFR* amplification. We found that 85% of the tumors displayed increased activation of at least one receptor compared to normal brain tissue. The
prerequisite receptor activation for tyrosine kinase inhibitors to be potentially useful is hereby shown to exist in a majority of glioblastomas.

Using an internal standard we divided phosphorylation of receptors into low (including cases with no detectable signal), moderate or high. Tumors were found to have high activation of either the PDGFRα or EGFR, or low to moderate activation of both receptors. Only one out of twenty-seven tumors analyzed had high activation of both receptors. Since functional studies have not been made on this material yet, it is difficult to say what level of phosphorylation is important for a cellular response. So, if tumors instead were stratified into groups with either low phosphorylation or moderate to high phosphorylation of either receptor, a different pattern appeared. Forty-one % had activation of both receptors, 33% had only the EGFR activated, and 7% had only PDGFRα activation. Regardless of criteria used for defining the subgroups, the characterization of tumor material prior to or in conjunction with treatment would be necessary to evaluate therapeutic effect.

The patients in this study had no known prior glioma and would clinically be considered to have primary GBM. With this in mind we found a larger group of tumors with TP53 mutation than expected (Watanabe et al., 1996). TP53 mutation did not correlate to age, EGFR amplification, or phosphorylation of either receptor. Although TP53 mutation has been shown to correlate with the clinical category secondary GBM, LOH on chromosome 17 may more be more useful to genetically separate subgroups (Hermanson et al., 1996). EGFR amplification correlated, not surprisingly, with high EGFR activation. Still, if EGFR amplification were used to identify tumors suitable for EGFR inhibitors, several tumors also having high phosphorylation levels would be missed. If on the other hand EGFR expression were used, several tumors may not respond due to lack of dependency on EGFR activity.

The question of heterogeneity is ever present in the analysis of GBM: Is the part analyzed representative for the entire tumor? The genetic analysis performed on microdissected material may, though guaranteed to consist of tumor cells, be biased by the small amount of tissue studied in this respect. The protein analysis is less likely to be flawed by microscopic, intercellular variations because of the relatively large tumor samples used and repeat analysis of primary tissue. Still, regional variations from one side of the tumor to the other cannot be excluded.
The immunoblot method used for the protein analysis in this paper would be too costly and consume too much material to use in clinical practice. Analysis of receptor phosphorylation would probably be most efficient with phospho-specific receptor antibodies on frozen or paraffin embedded sections. Due to variations of specificity of some inhibitors for the wild type and truncated EGFR, antibodies recognizing the latter ought also be used.

Receptor targeting therapies potentially effective in a subgroup of glioblastomas may be discarded for lack of response in the group as a whole if receptor activation status is not taken into account. Determining receptor activation status in tumors may also be done to design a receptor inhibitor combination treatment most likely to be effective for the individual patient.
Summary

The ultimate goal of all research regarding glioblastoma multiforme is to find a cure for this fully-fledged malignancy. Countless strategies have been tried with poor results. By dissecting the mechanisms behind its development and understanding what makes it grow, there are hopes to find a possible weakness, a soft belly, which could be used as a target for new types of therapy. The work in this thesis has made a small contribution to the understanding of some of the many aberrations seen in the human tumor.

In paper I, it was shown for the first time in an animal model that uncontrolled PDGF stimulation can play a major part, not only in growth of brain tumors, but also in their development. Cells from tumors induced by the MMLV/PDGFB virus was in paper II shown to depend on PDGF stimulation for continued growth in vitro.

In paper III, tumor development in the MMLV/PDGFB model was shown to be potentiated by loss of p53 and Ink4a-Arf, tumor suppressor genes often deleted in glioblastoma multiforme. This supports earlier studies identifying cooperative effects of PDGF signaling and Ink4a-Arf loss in tumor development, as well as models showing complementary effects of other forms of mitogenic signaling and p53 loss. In this study we see the specific cooperation of uncontrolled PDGF signaling and loss of p53, changes seen in secondary glioblastomas, for the first time. Attempts were made to identify the point(s) of interaction between increased mitogenic signaling and loss of these central tumor suppressors. Indications that facilitated signaling through the PI3K/Akt pathway, due to lower levels of PTEN, plays a part were seen.

Characterization of the activation status of the two growth factor receptors most commonly overexpressed in glioblastomas, the EGFR and PDGFRα, has been made, as a first step to determine if GBMs exhibit a functional dependency of their activation. Large variations in receptor activation that not always were related to variations in expression were observed. Results indicate that activation status may need to be taken into consideration when evaluating therapeutic studies aimed at a specific receptor. A continuation of
this preliminary study is ongoing. Tumor biopsies have been directly transplanted from patient to immunodeficient mice in order to test the efficiency of new, and already established, receptor inhibitors. Preliminary results indicate that response of xenotransplanted tumors to an EGFR inhibitor (PKI166, Novartis) is related to EGFR activation. Cell lines from a number of the glioma biopsies studied above are also being characterized regarding dependency on autocrine PDGF stimulation by a collaborating group.
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References


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distinct regions on the short arm of chromosome 17 associated with tumorigenesis of human astrocytomas. Proc Natl Acad Sci U S A 86, 7186-7190.


phosphatidylinositol 3'-kinase upon its association with pp185 (IRS-1) in intact rat livers. J Biol Chem 267, 17483-17486.


gliomas is invariably associated with gene amplification. Proc Natl Acad Sci U S A 84, 6899-6903.


