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MODELING PDGF-DRIVEN GLIOMAGENESIS IN THE MOUSE

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ABSTRACT

Gliomas are the most common form of brain tumors, with glioblastoma being the most aggressive form. Glioblastoma is characterized by a number of genetic aberrations, among them amplification and overexpression of platelet-derived growth factor receptor alpha (PDGFRA) that sometimes occurs together with inactivating mutations or loss of the tumor suppressor p53 (TP53). The infiltrative nature and rapid growth of glioblastoma make it incurable despite extensive treatment. A better understanding of the molecular genetic defects underlying brain tumor development is necessary in order to design novel and more efficient therapies.

In the present study we investigated how the combination of increased growth factor signaling and p53 loss induces brain tumors. We generated two transgenic mouse models overexpressing PDGF-B or the long isoform of PDGF-A under the glial fibrillary acidic protein (GFAP) promoter. Thus, the transgene is active in neural stem cells and astrocytes, cells that normally express GFAP.

We demonstrate that overexpression of PDGF-B on its own did not trigger brain tumor development. However, when the PDGF-B transgenic mice were crossed onto a Trp53 null background, malignant tumors resembling human glioblastoma appeared at the age of 2-6 months. These tumors displayed histopathological features of human glioblastoma with integrated vascular proliferations expressing PDGFR-β, glial tumor cells expressing PDGFR-α, pseudopalisading necrosis and abnormal cell nuclei.

The changes in the brains of PDGF-B/Trp53 null mice were evident long before tumors formed. We found increased numbers of PDGFR-α expressing cells, distorted vasculature, with prominent PDGFR-β expression in areas where brain tumors later occurred. In addition, neurosphere-forming cells were situated in more widespread locations compared to wild type (wt) mice.

In contrast to PDGF-B transgenic mice the overexpression of PDGF-A_L led to an early lethality of the mice. We detected increased numbers of undifferentiated glial cells and in a few mice neoplastic glioma-like lesions.

In summary, these studies provide new insights into the role of excessive PDGF exposure during brain tumor development.
LIST OF PUBLICATIONS

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

GFAP promoter driven transgenic expression of PDGFB in the mouse brain leads to glioblastoma in Tpr53 null background
GLIA. 2009; 57; 1143-53.

Brain abnormalities and glioma-like lesions in mice overexpressing the long isoform of PDGF-A in astrocytic cells

III. Hede SM, Nazarenko I, He X, Hedrén A, Andrae J, Nistér M.
Stem cells and vessels in pretumorigenic mouse brain
Manuscript.
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<tbody>
<tr>
<td>AII</td>
<td>Diffuse astrocytoma (WHO grade II)</td>
</tr>
<tr>
<td>AA</td>
<td>Anaplastic astrocytoma (WHO grade III)</td>
</tr>
<tr>
<td>AO</td>
<td>Anaplastic oligodendroglioma (WHO grade III)</td>
</tr>
<tr>
<td>AOA</td>
<td>Anaplastic oligoastrocytoma (WHO grade III)</td>
</tr>
<tr>
<td>ARF</td>
<td>Alternative reading frame protein (p14/p19Arf)</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>FGF2</td>
<td>Fibroblast growth factor 2</td>
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<tr>
<td>GB</td>
<td>Glioblastoma (WHO grade IV)</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>INK4</td>
<td>Inhibitor of CDK4</td>
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<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
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<tr>
<td>IDH1</td>
<td>Isocitrate dehydrogenase 1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDM2</td>
<td>Mouse double minute 2</td>
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<tr>
<td>MTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NG2</td>
<td>Chondroitin sulfate proteoglycan 4/Cspg4</td>
</tr>
<tr>
<td>NSC</td>
<td>Neural stem cell</td>
</tr>
<tr>
<td>OA</td>
<td>Oligoastrocytoma (WHO grade II)</td>
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<tr>
<td>OII</td>
<td>Oligodendroglioma (WHO grade II)</td>
</tr>
<tr>
<td>OPC</td>
<td>Oligodendrocyte progenitor cell</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PLGF</td>
<td>Placental growth factor</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RB</td>
<td>Retinoblastoma 1</td>
</tr>
<tr>
<td>RCAS</td>
<td>Replication competent ALV splice acceptor</td>
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<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<td>--------------</td>
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</tr>
<tr>
<td>SGZ</td>
<td>Subgranular zone</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
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<tr>
<td>TP53</td>
<td>Tumor suppressor protein 53</td>
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<tr>
<td>TV-A</td>
<td>Receptor for subgroup A avian sarcoma and leucosis virus (ASLV-A)</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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1 INTRODUCTION

1.1 CANCER

The development of a multicellular organism begins with a single cell, which gives rise to all cells constituting the living body. Many of these cells retain the ability to divide and grow long after development is completed, which is necessary in order to preserve adult tissue. The process of cell division is normally strictly regulated. However, in the case of cells disobeying the normal growth control program, the development of a tumor may occur.

Step by step these rogue cells acquire properties, which enable transition into cancer. These properties are described by Hanahan and Weinberg as hallmarks of cancer: support of chronic proliferation, eluding growth suppressors, avoiding cell death, promoting angiogenesis, limitless replicative potential, ability to invade, evading immune response, and adjustment of energy metabolism (Hanahan D. 2011). Tumor formation is a continuous process where also normal cells are constantly recruited to contribute to the creation of a tumor microenvironment. As an end result, a tumor does not only consist of tumor cells, generally it is heterogeneous. Heterogeneity makes therapy challenging as each tumor type requires specific treatment and different tumors of the same type respond to treatment in an individual manner.

Brain tumors are difficult to treat, as there are many subtypes of brain tumors with a wide range of biological aggressiveness. Gliomas are the most common primary tumors of the Central Nervous System (CNS) with glioblastoma (Andrae J) being the most aggressive. Each year 3 - 4 out of 100 000 adults are diagnosed with GB in most European countries, with 50% of the patients being over 60 years old (Ohgaki H 2005).

The expanding field of cancer research has given better insight into the key mechanisms behind tumorigenesis and significantly improved cancer drug discovery. However, prognosis for the patients with aggressive brain tumors is still very poor, which forces us to further characterize these tumors in order to identify new potential molecular targets for therapy.
1.2 PDGF/PDGFR

Platelet-derived growth factor (PDGF) was first discovered more than three decades ago as a serum growth factor for fibroblasts, smooth muscle cells and glial cells (Kohler N 1974; Ross R 1974; Westermark B 1976). It was originally identified as a disulphide-bonded dimer of two chains, PDGF-A (Betsholtz C 1986) and PDGF-B (Johnsson A 1982) and only later two additional genes and corresponding proteins were discovered PDGF-C (Li X 2000) and PDGF-D (Bergsten E 2001). At present, PDGF genes and polypeptides belong to the evolutionary conserved family of structurally and functionally related PDGF/VEGF growth factors. PDGF polypeptides can assemble into disulphide-bonded dimers: PDGF-AA, -AB, -BB, -CC and -DD. The PDGF-AB heterodimer is rarely found in vivo. Endogenous expression patterns of PDGF-A and PDGF-B generally do not overlap, which supports the fact that PDGF-AB heterodimers are infrequent (Hoch RV 2003). PDGF-B is mainly expressed in vascular endothelial cells, neurons and megacaryocytes, while PDGF-A and PDGF-C are expressed in epithelial cells, neuronal progenitors and muscles. PDGF-D expression patterns are less well known, but the protein is found in fibroblasts and smooth muscle cells (SMCs) (Andrae J 2008). The mammalian PDGF genes are situated on different chromosomes and have independent transcriptional regulation. However, the overlapping expression pattern of PDGF-A and PDGF-C suggests the possibility of common transcription regulatory mechanisms (Andrae J 2008).

All PDGFs have a highly conserved growth factor domain, called the PDGF/VEGF homology domain. This domain is involved in inter- and intra-disulphide binding of the PDGFs (Fredriksson L 2004). In order to be activated a short N-terminal extension present in PDGF-A and PDGF-B chains has to undergo intracellular proteolytic processing. PDGF-C and PDGF-D have a distinct protein domain as part of their N-terminal extension, called the CUB domain (Fredriksson L 2004). CUB domain prevents ligand - receptor binding until cleaved and activated by extracellular proteases (Bergsten E 2001; Li X 2000). The C-termini of PDGF-C and -D lack amino acid sequence extensions, while both PDGF-A and -B have a stretch of basic amino acids, that are mainly involved in extracellular matrix binding (LaRochelle WJ 1991; Ostman A 1991). The C-terminal extension consists of 18 amino acids, with a high proportion of lysine and arginine (Bonthron D 1992; Johnsson A 1984; Rorsman F 1992). There are two functionally distinct isoforms of the A-chain due to alternative splicing of exon 6, which encodes this C-terminal stretch; the short form of PDGF-A (PDGF-A_6) lacks the positively charged retention motif, and is freely diffusible, while the long form of PDGF-A (PDGF-A_1) can attach to extracellular matrix with the help of its C-terminal tail (Andersson M 1994; Heldin CH 1999a; Rorsman F 1988). The exact role of PDGF-A_1 and how it functionally differs from the shorter isoform is not well understood.

The five dimeric PDGF ligands act via two receptor tyrosine kinases (RTKs) PDGFR-α and PDGFR-β (Figure 1). PDGFRs have a common domain structure, including five extracellular immunoglobulin (Ig) loops and a split intracellular tyrosine kinase domain (Andrae J 2008). As a result of ligands binding to tyrosine kinase receptors, homo- and heterodimerization of the receptors occurs, which in turn leads to transphosphorylation of the intracellular domains and subsequent activation of intracellular signalling
pathways. The ability of the five different dimeric ligands to bind and activate the receptors varies as summarized in Figure 1.

![Figure 1](image-url)  
**Figure 1.** Receptor binding specificity of five dimeric PDGF ligands. *Ligand DD can activate αβ with lower specificity.

### 1.2.1 The role of PDGF during development

Ever since the first discovery of PDGFs, their functions have been extensively studied. Cell culture-based assays revealed their involvement in driving cellular responses, including survival, proliferation and migration. To study these cellular responses *in vivo* a large number of gain- and loss-of-function mutations in PDGF and PDGFR genes have been created in mice.

Analyses of PDGF-B, PDGFR-α and PDGFR-β knockout mice revealed severe phenotypes, lethal at embryonic stages (Betsholtz 2004; Levéen P 1994; Soriano 1994; Soriano 1997) while PDGF-A and PDGF-C knockout mice are lethal with some variability in time point depending on the genetic background of the mice (Boström H 1996; Ding H 2004).

Developmental defects found in these knockout mice underscore the importance of PDGF ligands and receptors in normal development. PDGF-B promotes proliferation of vascular smooth muscle cells (vSMCs) in angiogenesis and stimulates pericytes. PDGF-B is also involved in formation of glomeruli of the kidney (Lindahl P 1998; Lindahl P 1997a). PDGF-A is important in the normal development of lung alveoli (Boström H 1996; Lindahl P 1997b), intestinal villi (Karlsson L 2000), mesenchymal dermis, hair follicles (Karlsson L 1999) and in spermatogenesis (maybe also PDGF-C) (Gnassi L 2000). PDGF-C plays an important role in palate formation (maybe also PDGF-A). That similar phenotypes are observed in PDGF-A and -C knockout mice is not surprising, since both proteins act through the same receptor and have somewhat overlapping expression patterns (Ding H 2004; Ding H 2000). The function of PDGF-D in development remains unknown.
Studies in genetically modified mice, where the cytoplasmic signaling domains of the two PDGF receptors have been swapped, demonstrated that PDGF receptors are partly interchangeable during development and mediate very similar cellular responses. However, the PDGFR-β seems to have a more important intracellular signaling capacity in the vasculature, since mice with an introduced PDGFR-α signaling domain exhibited vascular defects (Klinghoffer RA 2001).

The presence of PDGFs is also important in adulthood, as they are involved in wound healing. However, excessive or aberrant expression of PDGFs can lead to pathological responses such as atherosclerosis, fibrosis and tumorigenesis (Andrae J 2008).

1.2.2 The role of PDGF in the central nervous system

Some insights into the role of PDGFs in the CNS were obtained before genetic mouse models were available. A series of in vitro studies determined that PDGF-A is expressed by neurons and astrocytes (Fruttiger M 2000; Noble M 1988; Yeh HJ 1991) and acts as a mitogen for oligodendrocyte progenitor cells (OPCs) (Noble M 1988; Raff MC 1988; Richardson WD 1988). Oligodendrocytes differentiate postnataly from PDGFR-α positive OPCs. PDGFR-α signaling is not required for OPCs specification, but continued proliferation and migration in CNS depends on PDGF-A signaling through PDGFR-α (Calver AR 1998; Fruttiger M 1999). In the absence of PDGF-A, postnatal surviving mice develop tremor due to severe hypomyelination (Calver AR 1998; Fruttiger M 1999). Similar phenotype is also observed for a PDGFR-α signaling mutants (Klinghoffer RA 2002). The amount of PDGF-A supply controls the number of OPCs not only during embryogenesis, but also in the adult brain (van Heyningen P 2001; Woodruff RH 2004).

Recent in vitro experimental data demonstrated the ability of PDGF-A to induce embryonic Nestin+ neural progenitor cells towards becoming NG2+ oligodendrocyte precursors (Hu JG 2008). In addition, direct stimulation with PDGF-A in the adult subventricular zone (SVZ), induces PDGFR-α positive neural stem cells to give rise to oligodendrocytic lineage, but not neuronal lineage cells (Jackson EL 2006; Menn B 2006).

Our knowledge on the role of PDGF-B and PDGFR-β in CNS is mainly based on their expression patterns in the brain. PDGF-B is present in embryonal as well as in adult neurons (Sasahara M 1991). PDGFR-β expression was detected in neurons and PDGF is involved in mediating neuroprotective functions after injury (Egawa-Tsuzuki T 2004; Ishii Y 2006; Smits A 1991). PDGFR-β is also found on fibroblasts and on pericytes (Hellström M 1999; Smits A 1989).

1.3 P53 AND APOPTOSIS

P53 was originally discovered while studying tumor viruses as a SV40LT-bound protein (Lane DP 1979; Linzer DI 1979). First it was considered to be an oncogene due to its ability to transform cultured cells when added together with the Ras oncogene (Eliyahu D 1984; Jenkins JR 1984). However, later studies revealed that p53 could
inhibit transformation and since then, p53 is known as a tumor suppressor gene (Finlay CA 1989). P53 nowadays is commonly referred to as the “guardian of the genome”. p53 is used as an alarm whenever a cell is exposed to various types of physiological stress or regulatory malfunctions to protect the organism from cancer. As a result of p53 activation a number of biological responses occur, such as cell cycle arrest, apoptosis, senescence or differentiation (Vousden KH 2009). Increased p53 levels can also induce proteins important for DNA repair. p53 reacts to hypoxia and lack of nutrients by inhibiting mammalian target of rapamycin (mTOR) or altering glucose uptake (Vousden KH 2009). Recently p53 has been implicated in regulation of self-renewal, symmetric division, quiescence, survival and proliferation in neural (Meletis K 2005), mammary (Cicalese A 2009), hematopoetic (Liu Y 2009a; Liu Y 2009b) and embryonic stem cells (Lin T 2005).

The p53 gene is located on chromosome 17p13.1. The protein has five functional domains; the transactivation domain (TA), situated in the N-terminus and needed for transcriptional activity and binding to MDM2 (Alarcon-Vargas D 2002; Unger T 1993); the proline-rich domain (PRD), responsible for p53 stability and shown to be important in inducing apoptosis (Toledo F 2007); the DNA binding domain (DBD), commonly mutated in a variety of human cancers and required for specific DNA binding (Soussi 2007); the C-terminal oligomerization domain that controls correct configuration of p53 (Chène 2001); and at the very end of the C-terminus a 30 amino acid long stretch that regulates DNA binding and transcriptional activity of p53 (Wolkowicz R 1997).

p53 is a very unstable protein and usually undergoes rapid proteasome mediated-degradation. However, p53 is stabilized whenever DNA damage or other types of physiological stresses occur. Protein levels are controlled by two critical upstream regulators, MDM2 and p19ARF. p53 has the ability to act as a transcription factor and enhance or repress the transcription of many hundreds of target genes (el-Deiry WS 1992), and it can indirectly regulate the function of thousands of genes (Wang L 2001).

Apoptosis is a complex and very important cellular response program, which can be activated by increased levels of p53, resulting in transcriptional activation of PUMA (Yu J 2005). Loss of apoptosis allows a cancer cell to overcome different cell-physiological stresses, such as signaling imbalance, and DNA damage. Cancer cells are creative in their ways to inactivate the mechanisms of apoptosis, for example by increasing the levels of anti-apoptotic Bcl-2-related proteins, changes in upstream regulators of p53, and methylation of the p53 promoter.

1.4 GRADING OF TUMORS OF THE CENTRAL NERVOUS SYSTEM ACCORDING TO THE WORLD HEALTH ORGANIZATION

Gliomas are the most common primary tumors of the CNS mainly affecting adults. Gliomas are categorized into astrocytomas, oligodendroglomas, oligoastrocytomas and ependymomas, reflecting their histological appearance. In a clinical setting, the grade of the tumor is a very essential factor to help predict the outcome of the patient and the choice of therapy. According to the World Health Organization (WHO) grading scale
for the tumors of the nervous system, lesions with low proliferative activities are
denoted as grade I, quite often curable upon surgical removal. Once the lesion is
infiltrative it is designated as grade II. Tumors of this grade can progress to higher
grades. Grade III are lesions with accelerated mitotic activity and nuclear atypia. Grade
IV is considered to be the most malignant grade with fatal outcome. These tumors often
present with infiltration into surrounding tissue, high mitotic activity and necrotic
areas (Louis DN 2007).

1.4.1 Astrocytoma

Astrocytomas are composed of tumor cells with histological characteristics of
astrocytes. The malignancy subtypes are pilocytic astrocytomas (PA, WHO grade I),
diffuse astrocytomas (AII, WHO grade II), anaplastic astrocytomas (AA, WHO grade
III) and glioblastoma (GB, WHO IV), with glioblastoma being the most common and
aggressive type (Louis DN 2007).

Astrocytomas are diagnosed based on histological findings, which include fibrillary
neoplastic astrocytes with distinct nuclear characteristics enclosed in a loosely
structured tumor matrix. The astrocytic nucleus is elongated and has an evident
nucleolus. The most common histopathological features of pilocytic astrocytomas are
Rosenthal fibers and hyaline droplets. It is a relatively defined tumor, which occurs
mostly in young adults. Diffuse astrocytomas are described as slowly growing tumors
with a high degree of cellular differentiation. A moderate increase in cellularity, nuclear
atypia and often absence of mitotic activity are the most common features of AII. As
well as the pilocytic astrocytomas, diffuse astrocytomas affect young adults. Diffuse
astrocytomas may progress to a more malignant grade. Once significant mitotic activity
is detected in a tumor, it is defined as an anaplastic astrocytoma grade III. AA can also
arise de novo. With progressive anaplasia, morphology of the nucleus becomes more
atypical, with a more prominent nuclear size, shape and number variation. Once
microvascular proliferation and necrosis are found in the tumors, they are upgraded to
glioblastoma grade IV. Two different types of GBs have been described, primary and
secondary. When tumors evolve from lower-grade tumors to higher-grade tumors, they
are called secondary GB. However, the majority of glioblastoma tumors develop de
novo, with no previous history, and are referred to as primary glioblastomas (Louis DN
2007). The primary and secondary GBs exhibit the same histopathological
characteristics, even though they differ in both genetic changes and clinical history
(Ohgaki H 2007). Glioblastomas have been shown to manifest themselves at any age,
but preferentially affect adults in the age range between 45 and 75 years old.
Interestingly, primary GBs are more frequent in older individuals, whereas secondary
GBs occur in middle-aged patients, and demonstrate longer survival (Louis DN 2007).

1.4.2 Oligodendroglioma

Oligodendrogliomas are comprised of tumor cells with histological features resembling
oligodendrocytes. The subtypes are oligodendroglioma (OII, WHO grade II) and
anaplastic oligodendroglioma (AO, WHO grade III). In general oligodendrogliomas are
slowly growing with relatively long survival times, affecting mostly middle-aged adults
(Louis DN 2007).
Oligodendroglioma grade II has moderate cellularity and display distinct histological features of monomorphic cells with round nuclei and perinuclear halos, on paraffin sections creating a “honeycomb” appearance. Additional characteristics are microcalcifications and a compact network of branching capillaries (resembling chicken wire). Scattered reactive astrocytes can be present in tumor tissue, but they can be distinguished from neoplastic astrocytes by their eosinophilic cytoplasm. Low mitotic activity in these tumors correlates with low abundance or absence of Ki67+ cells. On the other hand, anaplastic oligodendroglioma grade III is characterized by distinct mitotic activity, as well as microvascular proliferation and areas of necrosis, which indicates a less favorable prognosis for the patients with this tumor (Louis DN 2007).

1.4.3 Mixed glioma

The third group of brain tumors is mixed gliomas composed of a mixed tumor component resembling both glial lineages. The types are oligoastrocytoma (OA, WHO grade II) and anaplastic oligoastrocytoma (AOA, WHO grade III) (Louis DN 2007).

Oligoastrocytoma grade II is a diffusely infiltrating glioma with low or absent mitotic activity and moderate cellularity, with frequent microcalcifications and microcystic degeneration. Anaplastic oligoastrocytomas are characterized by presence of microvascular proliferation, high mitotic activity, cellularity, nuclear atypia and cellular pleomorphism. These tumors usually appear in middle-aged individuals. Prognosis for patients with oligoastrocytoma grade II is slightly better than it is for those with anaplastic oligoastrocytoma, who in turn have longer survival than patients with GB (Louis DN 2007).

1.5 GENETIC ALTERATIONS IN GLIOMA

A cancer cell evolves by both genetic and epigenetic changes helping the cell to escape normal mechanisms controlling cell survival, proliferation and migration (Figure 2). It is believed that a single alteration is rarely sufficient in turning a normal cell into a cancer cell. Most likely the process of tumorigenesis is a result of combined deregulations of complex pathways found to influence the control mechanisms. Molecular screenings of gliomas have uncovered an abundance of genetic and epigenetic changes.

1.5.1 Growth factor pathways

A frequent hallmark of malignant gliomas is activation of RTK signaling pathways, most commonly caused by epidermal growth factor receptor (EGFR) mutation/amplification or PDGFR amplification/overexpression. EGF and PDGF proteins exert their activity by binding and activating protein RTKs, which leads to receptor dimerization and transphosphorylation. Subsequently, activation of intracellular signaling pathways, such as PI3K/AKT and RAS/MAPK occurs, which in turn regulates survival and proliferation of the cell (Figure 2).
EGFR signals via RAS and Phosphatidylinositol 3-kinase (PI3K) pathways, stimulating cell division, survival and invasion. EGFR is frequently found to be amplified, mutated or rearranged in GBs. These alterations are closely related to reduced patient survival. Amplification of the EGFR gene is found in about 43% of primary GBs and is associated with EGFR overexpression. However, EGFR overexpression is rarely found in secondary glioblastomas (Ekstrand AJ 1991; Libermann TA 1984; Ohgaki H 2007; TCGA 2008; Wong AJ 1992). Furthermore, 70-90% of all GBs with EGFR overexpression have rearrangement of the gene (TCGA 2008). The most widespread mutated variant of EGFR is EGFRvIII, which contains a 267-base-pair (bp) deletion of exons 2-7 in the extracellular domain, resulting in ligand-independent activation of the receptor (Gan HK 2009; Ohgaki H 2007; Sugawa N 1990; Wong AJ 1992).

Hyperactivity of PDGF ligands and receptors are frequent events in human gliomas of all grades (Lokker NA 2002; Nister M 1988). PDGF ligands bind and activate receptor tyrosine kinases PDGFR-α and -β with different affinities. Binding leads to autophosphorylation of the receptors and activation of RAS and PI3K signaling pathways. Gliomas express PDGF-A and –B as well as the receptors (Di Rocco F 1998; Martinho O 2009; Nister M 1982; Nister M 1988). Their expression pattern in tumors suggests the presence of autocrine and paracrine stimulatory loops (Hermanson M 1992; Lokker NA 2002). However, amplification of PDGF and PDGFR genes is not as common as amplification of EGFR (Fleming TP 1992; Hermanson M 1996) and only occurs in 11% of GBs. However, this still makes PDGFR the second most frequent RTK gene amplified in these tumors (TCGA 2008). The activating gene rearrangements of PDGFR in GBs are very rare. Previously only two reports described an in-frame deletion of the Ig-like domain, PDGFRΔ8,9 mutant and a mutant in the C-terminal end of PDGFR (Kumabe T 1992; Rand V 2005). Recent sequencing analysis of GBs has found several point mutations in the Ig-like domain (Verhaak RG 2010) and another study discovered a gene fusion between the kinase insert domain receptor (KDR) (VEGFRII) gene and PDGFR (Ozawa T 2010). Ozawa and colleagues have demonstrated that the previously discovered PDGFRΔ8,9 mutant is present in 40% of GBs with PDGFR amplification.

Recently, alterations in other RTKs have been reported, including ERBB2/HER2 mutations and MET amplifications in 8% and 4% of GBs analyzed, respectively (TCGA 2008).

Increase in the tumor size results in hypoxia and induces a response from hypoxia-inducible factor 1 (HIF1), which in turn increases transcription of the vascular endothelial growth factor (VEGF) gene (Jensen RL 2006). VEGFs are often overexpressed in high-grade gliomas and their receptors VEGFR1/2 are important in blood vessels formation and tumor oxygenation (Grzmil M 2010).

High levels of active RAS have been reported in high-grade astrocytomas, but unlike in many cancers, mutated RAS is rarely present in malignant gliomas (2%) (TCGA 2008). RAS is a GTPase that stimulates both PI3K and MAPK pathways. Activity of RAS has been linked to the proliferation of astrocytoma cells (Guha A 1997). Neurofibromin-1 (NF1) is a tumor suppressor and negative regulator of RAS. Mutations of NF1 have been linked to the hereditary condition Neurofibromatosis type-1, where patients are
predisposed to glioma development (Sørensen SA 1986). *NF1* was recently found to be mutated in 18% of glioblastomas (TCGA 2008).

PI3K consists of a regulatory domain and a catalytic domain, which allow phosphatidylinositol-4,5-bisphosphate (PIP2) conversion to PIP3, in turn activating AKT. Mutations in the catalytic domain are commonly present in tumors and mutated *PIK3CA* is reported in 15% of glioblastomas (Samuels Y 2004). Activation of PI3K/AKT signaling can be achieved by loss of the tumor suppressor gene PTEN. PTEN is a direct antagonist of the activity of PI3K. *PTEN* loss is rare in low-grade gliomas, but mutations and deletions are found in 50% of high-grade gliomas (Knobbe CB 2003) and are associated with a poor patient survival. As a result, inactive PTEN leads to AKT hyperactivation, which in turn triggers downstream pathways by supporting cellular growth (through mTOR) and proliferation (through inhibition of GSK3-β) (Zundel W 2000). In addition, activated AKT plays an important role in inhibiting apoptosis, by directly activating MDM2, thus leading to the degradation of p53. AKT signaling can also activate several other pro-apoptotic proteins, like BAD, BAX or caspase-3 (Grzmil M 2010).

### 1.5.2 Cell cycle regulation

Loss of cell cycle regulation is another key alteration, found in gliomas (Figure 2).

P53 is a major regulator of multiple cellular responses including DNA damage, oncogene activation and hypoxia (Rich JN 2004). In GBs *TP53* is found to be mutated in 35% all cases (TCGA 2008). Until recently it has been described that somatic *TP53* mutations are more common in low-grade astrocytomas and secondary GBs, than in primary (Ohgaki H 2007; Watanabe K 1996). However, recent studies have confirmed that *TP53* mutations are also prevalent in primary GBs (Parsons DW 2008; Zheng H 2008). In addition chromosome 17p, where *TP53* is located, has been found to be an early and frequent target for loss of heterozygosity of both low-grade and high-grade gliomas (Ohgaki H 2004; von Deimling A 1992).

Inactivation of P53 can also occur through other mechanisms such as viral infection, loss of ARF and overexpression of *MDM2* (Soussi T 2007). The MDM4 and MDM2 proteins inhibit P53 from activating transcription and also target P53 for proteasomal degradation (Wade M 2010). *MDM2* overexpression is found in more than 50% of primary GBs (Biernat W 1997) and gene amplification is present in 10% of primary GBs, whereas amplifications of *MDM4* is found in 4% of GBs (Reifenberger G 1993; Riemenschneider MJ 1999).

Two different tumor suppressors p14ARF (p19Arf in mouse) and p16INK4a are encoded by *CDKN2A*. p14ARF binds to MDM2 and thereby inhibits P53 degradation. Loss of p14ARF is frequent and found in 76% of GBs (Nakamura M 2001).

The progression from G1 to S phase in the cell cycle is controlled by the p16INK4a/CDK4/pRb pathway (Figure 2). Genetic alterations involved in this pathway are found in 78% of glioblastomas (TCGA 2008). p16INK4a encodes a protein that by binding to CDK4/6 inhibits the CDK4/6 and CyclinD1 complex and thus inhibits the
G1 to S transition. p16INK4a is commonly deleted in 30% of gliomas (Ohgaki H 2007). Once normal p16Ink4a function is lost, the CDK4/6 and CyclinD1 complex activates the pRb protein and in turn causes the release of E2F. Amplification of CDK4 occurs in 14% of GBs. RB1 is mutated or deleted in 11% of GBs (TCGA 2008), but silencing and promoter methylation of RB1 is more common and found in 14% of primary and 43% of secondary GBs (Nakamura M 2001).

**Figure 2.** Molecular pathways involved in glioma. Modified from (Huse JT 2009; Rich JN 2004). Oncogenes are presented in purple and tumor suppressor genes in green.

### 1.6 MOLECULAR-GENETIC SUBCLASSIFICATION OF HIGH-GRADE GLIOMA

Earlier clinical and genomic studies of GB described two subtypes: primary and secondary. However, recent studies have provided more detailed information about the components important in glioma formation and progression and has identified new subgroups based on glioma molecular signatures (Brennan C 2009; Robertson T 2011; TCGA 2008).

The four suggested molecular-genetic subtypes of glioblastoma are:
1. The classical subtype is characterized by \textit{EGFR} amplification/overexpression and mutation, as well as loss and mutation of \textit{PTEN} and \textit{CDKN2A}. Activation of the Notch and Sonic hedgehog pathways is common for this subtype as well.

2. The mesenchymal subtype is characterized by loss or mutation of \textit{NF1}, \textit{TP53} and \textit{PTEN}. Overexpression of \textit{MET}, \textit{CD44} and activation of the TNF family and NF-kB are frequent events for this subtype.

3. The proneural subtype is characterized by \textit{PDGFRA} amplification and loss or mutation of \textit{TP53, CDKN2A, PTEN}. Mutation of \textit{IDH}1 and activation of PI3K and PDGFRA pathways are also frequent characteristics of this subtype. GBs of the proneural subtype have expression profiles corresponding to those of neuronal (\textit{SOX, DCX, etc}) and oligodendrocytic (\textit{PDGFRA, OLIG2, etc}) progenitor cells.

4. The neural subtype is characterized by overexpression or amplification of \textit{EGFR} and a gene expression profile of normal brain. The molecular-genetic changes described in the proneural subtype correspond to secondary GB. In addition to distinct genomic profiles, the classical, mesenchymal and proneural subtypes vary in their biological behavior and response to adjuvant treatments (Van Meir EG 2010).

Recent results from GB tumor sequencing studies revealed previously unknown important genetic changes. Spontaneous mutations of isocitrate dehydrogenase-1 and -2 genes (\textit{IDH-1} and \textit{IDH2}) appeared as strong prognostic indicators in anaplastic astrocytoma and secondary glioblastoma. For secondary GB, mutation of \textit{IDH-1} is linked to a median survival of 31 months compared to 15 months for the group of patients with wt \textit{IDH-1} (Robertson T 2011).

1.7 ANGIogenesis

The formation of atypical tumor vasculature and cell invasion are believed to be major factors responsible for the resistance of gliomas to treatment (Onishi M 2011). GBs are known to be among the most vascularised tumors. Growth of the tumor creates a demand for oxygen and nutrients, thus new blood vessels must be formed. The process of new blood vessels formation from pre-existing vessels is called angiogenesis. Glioblastoma angiogenesis is well-studied and tumors are known to have blood vessels with high permeability, increased diameter, thick basement membrane and highly proliferative endothelial cells (Lopes 2003; Onishi M 2011). Vascular homeostasis is normally achieved by a balance of pro- and antiangiogenic factors. Once the proangiogenic stimuli outweighs the antiangiogenic mechanisms, angiogenesis is activated. One of the most central proangiogenic factors is VEGF, which promotes angiogenesis, proliferation and migration of endothelial cells and permeability of blood vessels. Expression of VEGF and its receptors is significant in tumor angiogenesis and found in all gliomas (Chan AS 1998). VEGF-A is frequently expressed by tumor cells (Goldman CK 1993; Plate KH 1992). Normally VEGF-A binds to two RTKs, VEGFR-1 (Flt-1) and VEGFR-2 (KDR, Flk-1). The receptors, VEGFR-1 and VEGFR-2 are also overexpressed in endothelial cells of glioma (Plate KH 1994; Plate KH 1992).
The most effective activator of angiogenesis is hypoxia, which leads to stabilization of HIF-1α (Acker T 2004). This in turn leads to activation of DNA promoter regions known as hypoxia response elements (HREs). HIF-1α binding to HREs mediates transcription of more than a hundred genes that help the cells to deal with low oxygen levels by angiogenesis (VEGF and Ang1), cellular metabolism, survival/apoptosis (BNIP) and migration (MET, CXCR4) (Onishi M 2011).

1.8 NEURAL STEM CELLS

A common belief of classical neuroscience was that once development was completed, no new neurons were produced. However, this has changed and in the 1960s the first evidence of adult neurogenesis appeared from studies in rat brain (Altman 1962). During brain development, neuroepithelial stem cells situated in the ventricular zone give rise to neurons and glia. Glia consists of support cells, oligodendrocytes, which myelinate the axons of neurons and astrocytes, star-shaped cells with diverse functionality. Even though it was thought that neurogenesis is mostly completed by birth, it continues throughout life. Cells, commonly known as neural stem cells (NSCs), have a capacity to self-renew and differentiate along multiple lineages, contributing to ongoing tissue maintenance and regeneration in case of injury in the adult (Morshead CM 1994; Reynolds BA 1992). Since the first discovery, NSCs have been identified in several species, including humans (Doetsch F 1999; Kukekov VG 1999).

There are two regions where adult NSCs are known to reside, the SVZ of the lateral ventricle and the subgranular zone (SGZ) of the dendrite gyrus in hippocampus. The largest source of NSCs in the adult mammalian brain is the SVZ, which is described as a thin layer of proliferative cells lining the lateral wall of the lateral ventricle (LV) and separated from the ventricular lumen by a monolayer of ciliated ependymal cells (Doetsch F 1996). Mouse SVZ differs from human, as it lacks a hypocellular gap between the ependyme and astrocytic cells. Mouse SVZ contains more proliferating cells than the human and migratory chains that are formed by neuroblasts in mouse are not seen in humans (Lois C 1996; Quiñones-Hinojosa A 2006).

Adult NSCs are not unstructured undifferentiated cells, they show features of differentiated astrocytes and express glial fibrillary acidic protein (GFAP). However, only a small population of the astrocytes present in the adult brain have stem cell properties. This subpopulation of GFAP+ cells in the SVZ (B cells) produce a transit-amplifying cell population (C cells) that then give rise to the neuroblasts (A cells) that migrate to the olfactory bulb along the rostral migratory stream (Armstrong JF 1995) to mature into neurons (Doetsch F 1999; Garcia AD 2004; Lois C 1994). Most of the generated OPCs migrate into the neighboring corpus callosum (Menn B 2006; Nait-Oumesmar B 1999). Studies have shown that adult NSCs are derived from radial glia, the stem cells of the developing brain, which in turn are derived from the neuroepithelium, the earliest brain progenitors (Jackson EL 2008; Merkle FT 2004).

There are several architectural elements contributing to adult neurogenesis: NSCs situated near the cerebrospinal fluid (when in SVZ); widespread cell-to-cell interactions; close association with blood vessels; a rich extracellular matrix (ECM)
and specialized basal lamina (Doetsch 2003; Riquelme PA 2008). The endothelial cells of blood vessels release factors that are promoting the self-renewal of NSCs (Ramírez-Castillejo C 2006; Shen Q 2004).

In addition to GFAP expression, the type B cells are characterized by co-expression of Nestin (Doetsch F 1999). Sox2 expression is also found in NSCs of the SVZ (Ellis P 2004) and SGZ (Suh H 2007) and is required to maintain the immature stage of NSCs and to preserve their proliferation and generation of neurons (Favaro R 2009; Ferri AL 2004).

1.9 REGULATORY PATHWAYS OF NEURAL STEM CELLS AND THEIR RELATION TO BRAIN TUMORS

Studies on pathways involved in NSCs regulation revealed frequent involvement of these pathways in brain tumor development.

It has been demonstrated that SVZ stem cell astrocytes express PDGFR-α (Jackson EL 2006) and EGFR is expressed in type B and type C cells (Doetsch F 2002). This suggests that NSCs are able to respond to specific growth factor stimuli. Indeed, SVZ neural stem cells proliferate in response to both PDGF and EGF in vitro (Craig CG 1996; Doetsch F 1999; Erlandsson A 2001; Reynolds BA 1992). PDGF and EGF signalling pathways are often hyperactive in human gliomas of all grades. EGF is used in neurosphere cultures of NSCs to keep them in an undifferentiated state (Reynolds BA 1992). Infusion of EGF into the brain results in a significant amplification of endogenous SVZ precursor cells (Craig CG 1996; Doetsch F 2002; Kuhn HG 1997) and promotes oligodendrogenesis (Gonzalez-Perez O 2009). When active EGF receptor was retrovirally introduced into Ink4a/Arf-deficient mice, it caused de-differentiation of astrocytes. High-grade gliomas develop from both de-differentiated astrocytes and NSCs carrying the same genetic alteration (Bachoo RM 2002). PDGF also has a de-differentiating effect on mouse astrocytes (Dai C 2001) and infusion of PDGF-A into adult mouse lateral ventricle can induce the proliferation of PDGFR-α positive NSCs and lead to glioma-like reversible lesions. PDGFR-α is needed for oligodendrogenesis, but not neurogenesis (Jackson EL 2006).

p53 function is important during development of the CNS by inducing apoptosis of neurons and neural progenitors for adjustment of the cell number (D'Sa-Eipper C 2001) and a subset of Trp53 knockout mice develop exencephaly (Armstrong JF 1995; Sah VP 1995). Inactivation of the tumor suppressor gene TP53 is a common early event in human brain tumor development. P53 is expressed in the mouse SVZ and is involved in regulation of proliferation, apoptosis and self-renewing capacity of NSCs. In vitro, p53 deficiency in neurospheres results in reduction of apoptosis, increased self-renewal capacity and cell proliferation. Gene expression profiling identified several genes that were down-regulated in Trp53 null neurospheres, such as the cell cycle regulatory factors p21 and p27 (Meletis K 2005). P21 is an important negative regulator of the cell cycle and in preserving quiescence of NSCs (Kippin TE 2005). Olig2, which is a transcription factor, suppresses the expression of p21 in NSCs (Ligon KL 2007). Olig2 is commonly present in all types of glial tumors. Normally Olig2 is important for both
OPCs and oligodendrocytes development (Ligon KL 2004; Ligon KL 2006). Sox2 is another transcription factor that is important for NSC proliferation and inhibition of differentiation (Graham V 2003), Sox2 was found to be overexpressed in all glial tumors (Phi JH 2008).

It has been demonstrated that loss of Pten in Nestin positive NSCs results in abnormal brain development (Groszer M 2001). In neurospheres, deficiency of Pten leads to an increase in self-renewal by promoting exit from quiescent $G_0$ state and entry into the cell cycle (Groszer M 2006). $PTEN$ is found to be inactivated or mutated in about half of all high-grade gliomas (Knobbe CB 2003).

Some of the developmental pathways like those regulated by Notch and Shh are important in the stem cell niche. Activated Notch signaling affects both tumorigenesis and stem cell development (Hu YY 2011). Loss of Notch1 leads to a decrease in NSCs number and reduced proliferation (Hitoshi S 2002). Notch has also been shown to influence cell fate decisions throughout glial and neuronal development (Genoud S 2002). Shh signaling is active in adult SVZ and SGZ, where Shh expressing astrocytes induce neuronal progenitors to re-enter the cell cycle and generate new neurons (Jiao J 2008). It has also been shown that the SHH signaling pathway is active in human gliomas and correlates with the tumor grade.

1.10 CANCER STEM CELLS

1.10.1 Discovery of cancer stem cells

It has been shown that within some tumors there is a subset of cancer cells with stem cell-like capacities, such as unlimited self-renewal, which in turn preserves the tumor and gives rise to all the new cancer cells. Given these findings, the theory of so-called cancer stem cells (CSC) has been commonly used in the field of tumor biology (Reya T 2001). CSCs are also commonly referred to as tumor initiating cells or tumor propagating cells (Clarke MF 2006).

The first CSCs were described by John Dick’s lab, to be present in acute myelogenous leukemia (AML) (Bonnet D 1997; Lapidot T 1994). Following the first discovery, CSCs were identified also in solid tumors, including breast (Al-Hajj M 2003), brain (Singh SK 2004), ovary (Szotek PP 2006), colon (O’Brien CA 2007), pancreas (Hermann PC 2007), and prostate (Miki J 2007).

1.10.2 Brain cancer stem cells

A high rate of recurrence and resistance to treatment are major characteristics of malignant gliomas. The CSCs have the capacity to survive treatment and give rise to a new tumor with characteristics of the primary tumor (Bao S 2006). Since the adult brain has only a small population of proliferating cells that can accumulate several mutations needed for the transformation into a cancer cell, the adult neural stem cells/progenitor cells have been suggested as candidate sources for brain CSCs (Figure 3). The location of the tumors in the brain often corresponds to the areas where NSCs reside, in the SVZ and SGZ. In addition, there have been suggestions that tumorigenic
events can lead to de-differentiation of a mature cell and gain of stem cell properties (Bachoo RM 2002; Dai C 2001; Lindberg N 2009) (Figure 3), which can also explain concurrent expression of oligodendrocytic and astrocytic markers in gliomas.

Figure 3. Three possible ways for a cancer stem cell (CSC) to arise: a neural stem cell (NCS) acquires a mutation; a progenitor cell (PC) acquires two or more mutations; or a fully differentiated cell (DC) undergoes several mutations that transform and drive it back to a stem-like state.

A neurosphere assay was originally developed to study NSCs (Reynolds BA 1992), and this assay quickly became useful for isolating cancer stem cells from human malignant brain tumors. Just like in the original assay, subpopulations of cancer cells were able to form neurospheres. These cancer cells were positive for Nestin and CD133 and were able to self-renew and capable of multilineage differentiation. The ability of these cells to propagate the tumor in immuno-compromised mice was tested, showing that CD133+ cells were highly tumorigenic compared to CD133- tumor cells (Singh SK 2004). However, later studies have demonstrated that both CD133 positive and negative tumor cells are equally highly tumorigenic (Beier D 2007; Chen R 2010), indicating that CD133 is not a universal marker for brain CSCs. Many other markers of NSCs or progenitor cells are expressed by CSCs, such as Sox2, Bmi-1, Musashi (Hemmati HD 2003), Notch and Nestin (Fan X 2006).

Similarly to NSCs, brain CSCs are situated in a special microenvironment. CSCs in various human brain tumors are found in close proximity to blood vessels. CSCs have been shown to stimulate angiogenesis by secreting VEGF (Oka N 2007) and in return they have a constant supply of nutrients and oxygen (Calabrese C 2007). High-grade gliomas are characterized by microvascular proliferation and cancer cells in these tumors are found to migrate into surrounding tissue along the vessels. In addition to vasculature, other components of the specified niche, such as specific adhesion between CSCs and the surrounding cells or extracellular matrix (ECM) are important for CSC maintenance. L1CAM, a cell surface molecule that intervenes in cell-ECM and cell-cell interactions, was found highly expressed in CD133 positive glioma CSCs (Bao S 2008; Xie Z 2009).
1.11 USING ANIMAL MODELS TO INVESTIGATE GLIOMA

There is no doubt that knowledge gained from direct analysis of human tumors and \textit{in vitro} studies using cell lines has had an important role in tumor biology. However, these approaches have several limitations, for instance, the role of the microenvironment in tumor development and progression, angiogenesis, tumor cells invasion and response to a drug treatment are not easily modeled. To address these issues, many animal model systems have been created to study tumorigenesis. One of the most popular animal model systems is the mouse, since its genome is fully mapped, it is easily bred and genetically modified. There are however some differences between human and mouse tumorigenesis. For example, murine cells are more susceptible to immortalization and thus need fewer genetic or epigenetic changes for tumors to develop. Nevertheless, mouse models are still very useful tools to study brain tumorigenesis.

Several approaches have been used to create informative mouse models. One of them is the transplantation of cultured cells derived from human (xenografts) or rodent (allografts) brain tumors into immuno-compromised mice. One of the advantages of a xenograft model is the rapid appearance and reproducibility of a tumor. However, because tumor cells are transplanted from culture, tumor initiation cannot be studied using this model. In addition, tumors from cells cultured in the presence of animal serum tend not to have the histological appearance of human gliomas. Freshly dissociated human tumors that have been cultured in a system optimized for CSCs show an appearance and genotype more similar to the primary human tumor, when injected orthotopically (in the original position) (Lee J 2006). Using serial passages of these cells for injection can lead to a progression from a lower to a higher grade tumor (Wang J 2009). These models are widely used in preclinical trials. But as mice used for the model lack a functional immune system the model also differs from the natural way of tumorigenesis in humans and this can influence the result of drug testing. Also, transplanted human tumor cells frequently fail to survive and reproduce the tumor in a mouse. This could be caused by a difference between the human and mouse microenvironments. Tumors in this xenograft model are initiated by the injection of a large number of cells into the animal, which is a different case from spontaneous transformation that is supposed to occur in a single cell that obtains multiple changes to create a tumor. However, these models served their purposes in initial studies of the biology of gliomas and cancer stem cells (Dirks 2007; Dirks 2006; Singh SK 2003; Singh SK 2004).

An alternative mouse model approach is genetic modelling of tumorigenesis, by either deletion of tumor suppressor genes and/or overexpression of oncogenes. These genetically engineered mouse models (GEMMs) can develop histologically accurate tumors that enable specific genetic alterations to be linked to the tumor type. GEMMs show more similarities to the human situation and are well suited for preclinical studies. The use of GEMMs helped in understanding the role of each individual gene as well as the combination of several genes, involved in the process of tumorigenesis.

One of the first genetically modified mouse models appeared in 1984 and had an overexpression of a viral oncogene (Brinster RL 1984). With time genetic models have
advanced to more complex systems, where somatic cells could be targeted by retro-, lenti-, and adenoviral vectors to express oncogenes. Multiple genes can be deleted or introduced at any developmental time point and in specific cells, by using tissue-specific inducible promoters. More sophisticated methods such as tet-regulatable and cre-inducible alleles of genes can direct the duration, timing and tissue compartment of gene inactivation or expression. Transgenic mice are engineered by pronuclear injection of DNA into a fertilized mouse egg. But usually oncogene activation is not enough to transform normal cells into neoplastic, though it lowers their threshold for transformation and permits secondary genetic alterations to induce the tumor formation (Aguzzi A 1995). In a knockout model, created by homologous recombination in embryonic stem (ES) cells, germ line deletion of a gene that normally protects a cell from tumor formation can be achieved. And again, the loss of a tumor suppressor gene lowers the threshold of the cells for transformation and by additional cooperative alterations, a tumor is able to form.

1.11.1 PDGF-driven gliomagenesis in animal models

Several experimental models were created to induce gliomas by forced expression of PDGF. In general, overexpression of PDGF leads to excessive production of oligodendrocyte progenitor cells and mainly results in oligodendroglial tumors.

When a PDGF-B-encoding retrovirus was used for injection into newborn mice, highly malignant tumors developed in 40% of all animals with the mixed histology of GB or primitive neuroectodermal tumor (PNET). Expression of both PDGF-B and PDGFR-α was detected in tumors and led to the suggestion that autocrine stimulation is an important step in the development of brain tumors (Uhrbom L 1998; Uhrbom L 2000).

As another approach, the RCAS/TV-A model system was developed. This system is based on avian RCAS (replication competent ALV splice acceptor) retroviruses and transgenic mice expressing the receptor tv-a, under the control of a cell type specific promoter (Fisher GH 1999; Uhrbom L 2001). At present, there are three tv-a transgenic models, Ntv-a, Gtv-a and Ctv-a, which allow PDGF expression in Nestin, GFAP and CNPase expressing cells, respectively (Holland EC 1998a; Holland EC 1998b; Lindberg N 2009). Tumors in these models have oligodendroglial features, with some exceptions of mixed oligoastrocytomas in Gtv-a and Ctv-a mice (Dai C 2001; Lindberg N 2009). Newborn pups of all three transgenic lines were used to create gliomas in different locations such as cortex, cerebellum and brain stem. RCAS-PDGF injection into either the SVZ or the cortex of adult Gtv-a and Ntv-a mice results in gliomas with similar latency and frequency, however cerebellar and/or brain stem gliomas occur with longer latency compared to when injected in SVZ or cortex (Hambardzumyan D 2009). Tumor progression can occur during PDGF-induced gliomagenesis when combined with another genetic aberration such as loss of Inka/Arf, Trp53 or Pten (Hesselager G 2003; See WL 2010; Tchougounova E 2007). Also, an increased dose of PDGF-B in these mice causes shortened latency, increased cellularity and areas of necrosis and thus induces tumor progression. An elevated dose of PDGF-B in the brains of these mice mediates recruitment of vSMCs (Shih AH 2004).
A role of PDGF in the pathogenesis of spinal cord gliomas has been shown by overexpressing human PDGF-B (hPDGF-B) in Gfap expressing glia. Almost all transgenic mice developed spinal cord neoplasms resembling human mixed oligoastrocytoma (Hitoshi Y 2008).

In order to test the tumorigenic potential of adult glial progenitors, PDGF-B was retrovirally expressed in rat corpus callosum, leading to the infection and transformation of NG2 positive OPCs and development of GBs (Assanah M 2006). Injection of retroviral PDGF-B in newborn rats causes a shift in the differentiation fate of the NSCs, generating more PDGFR-α/NG2/Olig2 expressing OPCs, which do not differentiate into mature oligodendrocytes (Assanah MC 2009). It has been shown that excessive expression of PDGF-B in neural progenitor cells forces their respecification towards the oligodendroglial lineage with development of highly malignant oligodendroglial tumors in mice (Appolloni I 2009; Calzolari F 2008). Ventricular perfusion of the adult mice with PDGF-A leads to reversible glioma-like lesions of PDGFR-α, Nestin, and Olig2 expressing cells (Jackson EL 2006).

1.11.2 Inactivation of the p53 pathway in animal models

It has been suggested that inactivation of p53 is an early event in brain tumor formation, however it needs to be combined with another genetic alteration for a brain tumor to develop. Initial studies on four strains of Trp53 null mice concluded that p53 had no role in development (Donehower LA 1992; Jacks T 1994; Purdie CA 1994; Tsukada T 1993). Later findings revealed that a subset of Trp53 deficient embryos died during development. Their death was associated with defects in neural tube closure and excessive growth of neural tissue in the midbrain region (Armstrong JF 1995; Sah VP 1995). In general, Trp53 null mice have a short life-span, as they have an enhanced oncogenic potential and develop several tumor types, mostly sarcomas and lymphomas by six months of age (Donehower LA 1992). Trp53 heterozygous mice are also predisposed to tumorigenesis, but remain tumor-free for approximately nine months, before developing lymphomas and sarcomas at around eighteen months of age. Trp53 gain-of-function knock-in models develop carcinomas within one year rather than sarcomas, in contrast to Trp53 null mice (Liu G 2000).

The generation of Trp53 conditional knockout mice in brain tissue has been helpful in investigating the involvement of p53 in brain tumorigenesis together with other genetic alterations. For example, loss of p53 in astrocytic cells is not sufficient to develop a glial tumor (Marino S 2000), but this would require loss of another cell cycle regulatory factor, like pRb or excessive activation of growth factor signaling pathways (Furnari FB 2007).

1.11.3 Animal models with combined deregulation of growth factor signaling pathways and p53

Development of brain tumors with malignant phenotypes usually requires several genetic alterations. One such model for astrocytic glioma is created by combining NFI and Trp53 mutants, resulting in tumors of various histological grades (Huse JT 2009; Reilly KM 2000). Interestingly, tumors occurring in older animals were of higher grade, and similar to more slowly evolving secondary GB. A more recent pairing of a
Trp53 mutant allele with Nfl allele or their combined deletion in Gfap expressing cells led to complete penetrance of high-grade astrocytomas within 20-40 weeks (Wang Y 2009; Zhu Y 2005). However, tumors rarely developed when Nfl was lost before Trp53, indicating the importance of the initial loss of Trp53. High-grade tumors only appeared after the loss of Trp53 or at the same time with Nfl loss (Zhu Y 2005). Addition of a mutant Pten to this model led to a shorter latency and a higher grade of the appearing tumors (Kwon CH 2008). High-grade gliomas developed in mice where Trp53, Nfl and Pten were deleted in embryonic and adult Nestin expressing progenitors (Alcantara Llaguno S 2009).

Specific deletion of Trp53 and Pten in the mouse Gfap expressing cells generated tumors with a phenotype of high-grade malignant glioma, resembling primary GB in humans (Zheng H 2008).

Cre–LoxP-mediated inactivation of pRb and p53 tumor suppressor genes in the cerebellar external granular layer cells (LaRochelle WJ 2001) led to highly aggressive embryonal tumors of the cerebellum with typical features of medulloblastoma (Marino S 2000). In a recent study, the combined deletion of pRb and Trp53 or deletion of pRb, Trp53 and Pten led to PNETs (Jacques TS 2010). In another study the pRb pathway was inactivated in mature astrocytes by expressing truncated SV40T antigen (T121) under the GFAP promoter, and this resulted in astrocytomas within 300 days and with 100% penetrance (Xiao A 2002). Tumor progression was accelerated and shorter latency was observed in this model by the presence of Pten null heterozygosity (Xiao A 2005).

The role of RAS signaling in gliomagenesis has been studied in several mouse models. GFAP-v-src transgenic mice appeared to have nests of proliferating astrocytes that advanced into malignant astrocytoma of the brain and spinal cords (Weissenberger J 1995). Additional transgenic mice with constitutively active Ras under control of the GFAP promoter were generated. The grade of the tumors in these mice was directly correlated with Ras dosage. Mice with moderately elevated Ras levels developed astrocytomas of varying grades, while mice with a high expression of Ras died two weeks after birth with multifocal GB (Ding H 2001; Shannon P 2005). Constitutively active forms of K-Ras and Akt were transferred to Nestin and Gfap expressing progenitors using the RCAS/Tv-a system, causing development of high-grade astrocytomas in Ntv-a, but not in Gtv-a transgenics. In addition, activation of Ras or Akt alone is insufficient for tumor formation (Holland EC 2000). However, when Ntv-a and Gtv-a were crossed onto an Ink4a-Arf null background, following infection with RCAS-K-Ras resulted in brain tumors with sarcoma-like histology (Uhrbom L 2002; Uhrbom L 2005). In adult mice, lentiviral mediated introduction of H-Ras and Akt in the Gfap expressing cells of the SVZ and hippocampus led to grade III astrocytomas. More malignant, glioblastoma-like tumors appeared when adding a heterozygous Trp53 phenotype (Marumoto T 2009). Ras-driven gliomas can also be generated by loss of Pten (Hu X 2005).

Transgenic mice expressing v-erbB, a transforming version of EGFR develop low-grade oligodendrogliomas. However, v-erbB transgenic animals heterozygous for Ink4a-Arf or Trp53 developed high-grade tumors (Weiss WA 2003). Similar results
were shown in an earlier study where constitutively activated EGFR in *Ink4a-Arf null* NSCs and astrocytes led to high-grade gliomas (Bachoo RM 2002).

In summary, studies using genetically engineered animal models have illustrated the importance of PDGF, EGF, P53, RAS and PI3K pathways in brain tumorigenesis. Combined dysregulation of any two of these regulatory pathways leads to gliomagenesis.
2 AIMS OF THE PRESENT INVESTIGATION

The general aim of this thesis was to contribute to the understanding of the molecular mechanisms underlying gliomagenesis.

1. Paper I. By combining two common aberrations found in human gliomas, excessive expression of PDGF-B and loss of p53 in a mouse model we aimed to characterize the resulting brain tumor phenotype and compare it to human glioblastomas.

2. Paper II. The exact functional role of the long isoform of PDGF-A is not well characterized. By creating a transgenic mouse model with excessive expression of PDGF-A L in the brain we aimed to elucidate its role in brain development and gliomagenesis.

3. Paper III. By using the model generated in paper I we aimed to study cellular changes occurring in the brains of mice before the tumors developed, and to start investigating the effects caused by PDGF-B overexpression and loss of p53.

By generating these two transgenic models we aimed to draw conclusions regarding the functional differences between two members of the PDGF family in brain tumor formation.
3 RESULTS AND DISCUSSION

3.1 PAPER I: GFAP PROMOTER DRIVEN TRANSGENIC EXPRESSION OF PDGFB IN THE MOUSE BRAIN LEADS TO GLIOBLASTOMA IN A TRP53 NULL BACKGROUND

The genetic aberrations present in human tumors have been used to create animal model systems, which are important tools in studying the mechanisms behind tumor formation. These models may reveal which genetic changes are needed for tumor initiation and progression. This approach has helped define the main signaling pathways in glioma development (Holland 2001).

In this study we created a mouse model in which overexpression of PDGF-B was directed to cells of astrocytic lineage and NSCs by the human GFAP promoter. The 1.8kb GFAP promoter is active throughout the life of the mouse, starting as early as E8.5 (Andrae J 2001a). By X-gal staining we could easily track expression, as the transgenic construct also contained a beta-galactosidase reporter gene.

By using immunohistochemistry to detect the beta-galactosidase (β-gal) protein, strong expression of the PDGF-B transgene was detected in the lateral ventricle wall and roof of newborn mouse brains. In adult mouse brain, the expression was weaker, but the β-gal protein could still be detected in the lateral ventricle wall and in scattered astrocytes throughout the brain.

Mice overexpressing PDGF-B did not develop any brain tumors. They were crossed with Trp53 null mice to create the combined PDGF-B/p53null (B/p53null) offspring. In this way, two aberrations common in human gliomas were introduced in the mice. Mice of different genotypes were followed as long as 18 months for possible changes in phenotype. The Trp53 null mice developed lymphomas and sarcomas within six months and had no brain tumors, as previously described (Donehower LA 1992). However, mice from two transgenic lines with the combined genotype, B/p53null developed brain tumors at high rates, 68% and 43%. Tumors occurred at the age of 2-6 months and spread diffusely throughout the brain tissue as well as on the pial surface.

The strategy of overexpressing PDGF-B in mouse brain using retroviral systems has been used previously and led to induction of brain tumors in these animals (Appolloni I 2009; Shih AH 2004; Uhrbom L 1998). In these models, oligodendroglioma-like and glioblastoma-like tumors appeared within 12 weeks of injection. When in a Trp53 null background the brain tumors developed at a higher frequency and with a shorter latency than in the wt background. In our study, tumors only appeared when p53 was absent. Tumors in our model hardly displayed oligodendroglioma-like features. This discrepancy could be due to differences in targeted cells, dosage of PDGF-B as well as retroviral integration mutagenesis. In addition, although the transgene is active already in the embryo, the tumors only develop in adult mouse brain, which suggests that affected cells may need more time to proliferate and acquire additional genetic changes.
According to previous findings, human brain tumors show high levels of PDGFR-\(\alpha\), and PDGFR-\(\beta\), which is expressed on the pericytes surrounding the blood vessels (Hermanson M 1992). This was also the case in the PDGF-B tumors we generated. Moreover, the experimental tumors in our study are comparable to the human tumors with vascular proliferations, positive for PDGFR-\(\beta\). The majority of glioma cells expressed the transgene, this indicates the presence of both autocrine and paracrine stimulatory loops. Additionally, the tumors had some other glioblastoma-like features such as presence of palisading cells lining necrosis.

Large tumors in the brains of B/p53null mice had spread diffusely throughout the brain, occurring in the cerebellum, cerebrum, and brain stem. The tumors were found to express different lineage markers (Gfap, Nestin, Map2, TuJ1, Vimentin, F4/80 and CNPase) suggesting that the cell of origin could potentially differ between experimental animals, and also as the tumors had different locations in the brain. The cells of origin could possibly be migratory NSCs, making it possible for them to spread and form a tumor. A second option is that astrocytes de-differentiated and created a tumor. Interestingly, the neural stem cell marker Nestin was mainly present in the larger tumors. Another interesting observation was that Gfap expression was lost in some parts of the larger tumors, whilst transgene expression still remained present. Overall, some Gfap expressing cells were present in both small and large tumors, although, it is to some extent challenging to distinguish Gfap positive tumor cells from Gfap positive infiltrating reactive astrocytes in the tissue sections.

Small or early tumors in these mice were found in the lateral ventricle wall area and in association with glia limitans/pia, the areas where the GFAP promoter is known to be active. These early tumors arising from the ventricle wall may possibly represent a subpopulation of excessively proliferating NSCs in the SVZ, which could play a role in later malignant progression. A small fraction of Gfap expressing cells in the SVZ was previously shown to express PDGFR-\(\alpha\) and to form hyperplastic, tumor-like, infiltrating but reversible lesions in response to PDGF-A administrated via the ventricles (Jackson EL 2006). These lesions lacked vascular structures and integrated development of the tumor, which is in contrast to our model, where PDGF-B could possibly create response via both receptors \(\alpha\) and \(\beta\). It has been demonstrated that the absence of p53 induces a change in the behavior of NSCs in the SVZ by an increase in Gfap expressing astrocytic stem cells (Gil-Perotin S 2006). In addition, loss of p53 has been linked to increased self-renewal and proliferation of NSCs (Meletis K 2005).

As previously mentioned, transgene expression was relatively weak in the adult brain. However, comparing expression levels of \(\beta\)-gal in small and larger tumors revealed its strong presence even in the smallest tumors. \(\beta\)-gal expression in the larger tumors was versatile and the same variability in expressing cells was shown in tumors stained for PDGF-B, which could possibly mean that there are some cells forcing tumor growth by high levels of PDGF-B.

Thus, in this study sustained overexpression of PDGF-B in astrocytic cells leads to uncontrolled expansion of PDGFR-\(\alpha\) expressing cells and initiation of aggressive brain tumors in Trp53 null mice, mimicking the human situation in gliomagenesis. Most likely, these tumors were derived not only from the SVZ, but their wide distribution
may reflect the plasticity and de-differentiation potential of all astrocytic cells in the adult brain.

3.2 PAPER II: BRAIN ABNORMALITIES AND GLIOMA-LIKE LESIONS IN MICE OVEREXPRESSING THE LONG ISOFORM OF PDGF-A IN ASTROCYTIC CELLS

We examined the effects of PDGF-A_L overexpression in transgenic mice under the control of a human GFAP promoter. As in the previous model, this promoter allows us to direct the expression of PDGF-A_L to astrocytic cells and GFAP expressing NSCs throughout the developing and adult CNS. We detected expression of the transgene in newborn mice, with a strong staining throughout the brain by X-gal staining. In the adult brains we found particularly strong β-gal expression in the roof of the lateral ventricles, in corpus callosum, on the outer surface of the brain and in cerebellum.

These PDGF-A_L mice had significant skull enlargement at the approximate age of one and a half months. In total, 25 out of 26 mice were sacrificed or died due to neurological symptoms. Necropsy of these mice revealed an abnormal withholding of fluid in the subarachnoid space, with an unusually compressed and smooth brain shape created by the pressure. As there was only a slight change in the size of the ventricles in some of the brains, we excluded the possibility of hydrocephalus caused by blockage. Hydrocephalus in humans usually occurs due to a blockage in the flow of cerebrospinal fluid, which then accumulates in the ventricles and causes the expansion of the ventricles. Since the fluid in the heads of the mice was surrounding the brain, rather than being caught in the ventricles it could suggest that there was a problem with reabsorption of this fluid. The choroid plexus produces most of the cerebrospinal fluid, we also checked its appearance but found no changes.

We then performed more detailed analysis of the brains from PDGF-A_L mice. First, we stained sections with Hemotoxylin-Eosin and found certain areas with highly increased cellularity. These cells had small rounded nuclei, clear cytoplasm and were found in the wall and roof of the lateral ventricles, within the corpus callosum, in the medulla of the cerebellum and on the pia lining, where these cells formed outgrowths. Cells were also present in the white matter of the pons and in some cases also in the brain stem. Due to the presence of these cells in the cerebellum, its structure appeared to be largely disorganized. We confirmed our visual finding by counting the cells in different areas and comparing the numbers of cells with corresponding areas of the wt brains.

By staining with a Ki67 antibody we determined cell proliferation rates. As expected, in wt brains a small area of exclusively proliferating cells was found in the lateral ventricle wall. In contrast, all the areas in PDGF-A_L mice with excessive accumulations of cells displayed an increased percentage of Ki67 positive cells.

Next, by performing immunohistochemistry for β-gal we noted that the distribution of the extra cells overlapped with the transgene expression, which supports PDGF-A_L ability to induce cell expansion. Accumulation of cells in the mouse brain in response
to PDGF-A₅ stimulation was previously demonstrated in a study where PDGF-A₅ was directly injected into the lateral ventricles of mice (Jackson EL 2006). As a result they found fully reversible hyperplastic, infiltrating tumor-like lesions. In our study, the constant stimulation of mouse brains with PDGF-A₅ resulted in the brain lesions displaying features of neoplasia. Some of the mice had heavy diffuse infiltration of cells throughout the whole brain, comprising most white matter areas. The pial outgrowths were excessive cell rich masses with mitotic and atypical cells. More diffuse tumor-like areas were present also in the temporal lobe.

In order to determine which cells were responding to PDGF-A₅ stimulation, we performed immunohistochemistry for different cell type-specific markers. We first observed that in the areas of increased cellularity and positivity for β-gal there was strong Gfap expression. Both Gfap and β-gal expressing cells showed astrocyte-like morphology indicating that PDGF-A₅ was produced by astrocytic cells. Because PDGF-A₅ has a retention motif that helps it associate with the surface of the producing cell, we would expect that its site of action is rather limited. Observation of an increased number of PDGFR-α expressing cells in the areas of β-gal positivity could indicate that these cells are responsive to stimuli from PDGF-A₅. PDGFR-α is normally expressed by glial progenitor cells (Raff MC 1983; Raff 1989), and it has been previously described that PDGF-A is a potent mitogen of OPCs (Calver AR 1998; Fruttiger M 1999; Woodruff RH 2004).

To support the result of the presence of oligodendrocyte progenitor cells, we additionally stained sections for Olig2 and NG2 and observed a notable increase in expression of both markers in all areas with cell accumulation. The absence of oligodendrocyte protein CNPase suggested there were no mature oligodendrocytes. It has been demonstrated that PDGF-A can influence cell-fate decision of the cells situated in SVZ by inducing them to become OPCs rather than neurons (Jackson EL 2006).

Even more, it has been shown that PDGFR-α is also expressed by a subpopulation of SVZ B cells, which are also positive for Gfap. These cells are sensitive to alterations in the PDGF pathway and form hyperplasias in response to PDGF-A₅ stimulation, as mentioned previously (Jackson EL 2006). In our PDGF-A₅ transgenic mice with neoplastic lesions we found increased amounts of cells expressing PDGFR-α, Olig2 and Gfap, respectively. The increased proliferation was indicated by a Ki67 labeling index as high as 53% compared to only 1.4% in the corresponding area of the wt brain. The areas of neoplasia were closely associated with angulated and thick-walled capillary structures, strongly positive for PDGFR-β.

Interestingly, the majority of Olig2 and PDGFR-α positive cells in the examined brain sections did not express Gfap or the transgene marker. This difference could possibly be due to the Gfap protein being processed into several splice variants and the antibody used being unable to detect the variant present in NSCs. In humans, a GFAP-delta splice variant is specifically produced in neural stem and progenitor cells (van den Berge SA 2010). But the observation could also be due to a difference indicating that the transgene produced by NSCs/astrocytic cells stimulates OPCs in a paracrine fashion, or indirectly via changing the cell fate of the NSC/astrocytic progenitor.
We examined the cerebellum of the PDGF-\textsubscript{AL} transgenic mice. As mentioned earlier, the cerebellum showed abnormal morphology. Using the marker NeuN for mature granule cells, and Calbindin for Purkinje cells, we found that the layers of the cerebellar cortex preserved their correct order in relation to each other, despite the abnormal expansion of OPC-like cells into the granular layer.

In summary, this work demonstrates that overexpression of PDGF-\textsubscript{AL} can induce abnormal accumulation of immature OPC-like and astrocytic cells in the adult brain. Hyperactive production of PDGF-\textsubscript{AL} in the brain has distinct and important effects that are different from previously described studies on PDGF-\textsubscript{AS} and PDGF-B overexpression, and in some mice resulted in glioma-like lesions. Notably, PDGF-A is the predominant PDGF variant produced by human glioma cells (Nister M 1982; Nister M 1984) and PDGF-\textsubscript{AL} was originally detected and cloned from the same human glioma cells (Betsholtz C 1986).

### 3.3 PAPER III: STEM CELLS AND VESSELS IN PRETUMORIGENIC MOUSE BRAIN

In the first study we confirmed the contribution of two genetic changes in brain tumor formation, by overexpression of PDGF-B in \textit{Trp53 null} (B/p53null) mice. These mice with the combined genotype developed glioblastoma-like tumors at a high frequency. The goal of the present study was to see how both of these genetic changes co-operated to drive tumor development.

We compared the brains of B/p53null mice with brains from PDGF-B, p53null and wt mice before tumors occurred. First we studied the neural stem cells of all genotypes by neurosphere cultures and found them to be multipotent; they were able to differentiate into oligodendrocytes, astrocytes and neurons. We could detect the presence of the transgene by X-gal staining in PDGF-B and B/p53null neurospheres. Next, the growth rate was compared by measuring the size of spheres of each genotype. Absence of p53 was previously shown to increase self-renewal and proliferation of NSCs (Meletis K 2005). We found higher growth rate in both p53null and B/P53null compared to spheres from wt.

Brain tumors deriving from B/p53null mice develop in different regions of the brain as we demonstrated previously. These findings suggest that the tumor cell of origin could possibly be located in these different areas or the NSCs migrate out from the SVZ to form tumors. Tumor initiating cells and NSCs share common characteristics, we therefore tested the ability to establish neurosphere cultures from the different regions of the brain. In addition to the lateral ventricle wall, neurosphere cultures were prepared from corpus callosum, hippocampus, frontal (cortical) and basal (brain stem) subpial regions. We could establish cultures from the lateral ventricle wall of all four genotypes (wt, p53null, PDGF-B and B/p53null), but also from corpus the callosum of B/p53null brains. These neurospheres from the corpus callosum were later stained with X-gal and showed 100% positivity indicating transgene activity. In addition, we discovered high
secondary neurosphere forming capacity of B/p53null corpus callosum-derived spheres compared to spheres from the SVZ.

We then focused on the corpus callosum in the brains of all four genotypes and discovered an increase in total number of cells in B/p53null mice. These cells were not OPCs as immunohistochemistry against Olig2 demonstrated. Interestingly, in the PDGF-B genotype we found a significant increase in Olig2 expressing cells in the corpus callosum, but not in the B/p53null genotype. At the same time there was a slight increase in Gfap expressing cells in the B/p53null genotype, but not in the PDGF-B genotype. Next BrdU labeling experiments were performed to detect proliferating cells. We detected BrdU and Gfap double-positive cells in the corpus callosum of B/p53null brains, and we also discovered twice as many PDGFR-α positive cells in the corpus callosum of B/p53null mice when compared to wt mice.

Interestingly, different stem cell markers (Gfap, Olig2 and Sox2) that were present in the brain tumors of B/p53null mice were found to be co-expressed together with the transgene in isolated neurospheres. Neurospheres derived from the brains of all genotypes expressed Olig2, Gfap, Nestin and Sox2. As a next step we crossed the PDGF-B transgenic mice to a PDGFR-α/GFP receptor mice strain. This allowed us to visualize PDGFR-α expressing cells in neurospheres originated from the SVZ of PDGF-B/GFP and B/p53null/GFP mice. Immunofluorescence staining for β-gal indicated co-expression of the transgene with PDGFR-α in the neurospheres, but not necessarily in the same cells. This suggests that a PDGF-B autocrine, or local paracrine loop stimulates growth of the NSCs in the pre-tumor brains.

Brain tumor cells, obtained from the brain stem tumor of a B/p53null mouse could not form spheres. But similarly to neurospheres deriving from B/p53null non-tumorous brains, these tumor cells expressed β-gal together with stem cell markers Nestin, Olig2 and Sox2.

Even though we have a sustained increased supply of PDGF-B in the brains of transgenic mice from embryonic day 13.5, brain tumors appear only in the adult brain of B/p53null mice. This could be due to a slow progressive increase and accumulation of PDGF responsive cells in the brains of these mice. We previously noted an apparent increase in PDGFR-α positive, but not PDGFR-β positive cells in the subpial region of newborn B/P53null mice compared to the brains from wt mice. When looking at the subpial region in the brains of adult mice before tumor formation, we clearly observed an increased amount of PDGFR-α expressing cells, but also PDGFR-β expressing vessels. This was confirmed by counting positive cells in the different brain regions of the adult brain: SVZ, hippocampus, corpus callosum, frontal cortex and basal brain at the brain stem level.

One of the successful steps in tumor progression is the cells close proximity to blood vessels. High-grade gliomas are often characterized by PDGFR-β+ vascular proliferations (Hermanson M 1992). In our model, immunohistochemical stainings for PDGFR-β demonstrated an increased number of vessels in the B/p53null adult brains. On closer examination, these vessels were abnormal, with thicker walls and wider lumen and stronger PDGFR-β expression. Abnormal PDGFR-β expressing vascular
structures were also discovered in the retina of B/p53null mice. Expressing both
PDGFR-β and alpha smooth muscle actin (ASMA), these cells in the retina had
resemblance with pericytes, but had slightly different morphology and were not so
closely attached to the retinal blood vessels as normal pericytes.

In conclusion, our findings demonstrate how the combination of overproduction of
PDGF and absence of p53 results in an abundance of neural stem and progenitor cells
as well as altered vasculature. The fact that these changes were not present in the brain
of PDGF-B or p53null mice shows the importance of the combined effect of these
genetic changes in glioblastoma development.
4 SUMMARY

The capacity of different PDGF family members to facilitate tumor development has been in focus for intense investigations ever since the discovery of the growth factors and their identification in high quantities in GB cell lines and tissues. Very early on it was hypothesized that uncontrolled growth factor stimulation could play a role in tumor initiation.

In the present work we elucidate the role of two different members of the PDGF family with regard to tumorigenesis, PDGF-B and the long form of PDGF-A. We generated two different mouse models overexpressing either protein in all cells of the astrocytic lineage including NSCs.

Two common aberrations in human glioblastomas are overactive, PDGF signaling and lack of P53 function. By crossing PDGF-B transgenic mice onto a Trp53null background we could compare the brain tumors appearing in mice to the human situation.

Loss of p53 by itself does not lead to the brain tumor formation in our models, but it is an early event, which leads to increased proliferation and increased self-renewal of NSCs. By adding excessive expression of PDGF-B we induced NSCs properties, preserving their increased self-renewing and proliferating capacities. It is possible that an altered NSC niche can serve as the origin of gliomas. However, the tumors in our models develop in more widespread areas and were not located strictly in SVZ area, where NSCs reside. This could be due to migration of NSCs in PDGF-B/p53null mice from SVZ to other locations. Since the transgene is also expressed in mature astrocytes of the brain, the combination of p53 loss and overexpression of PDGF-B may induce astrocytes to de-differentiate and acquire self-renewing capacities similar to NSCs and to form tumors. To test the theory of origin of glioma cells from NSCs, we could combine hGFAPpPDGF-B mice with conditional knockouts of Trp53 in NSCs of the adult SVZ. By doing so, we could see if tumors would only be able to develop in the lateral ventricle wall or also in other areas.

PDGF-B/p53null mice were used to study the brain before tumor formation to define the changes occurring in the combined genotype and individually. By doing so, we could observe effects similar to alterations present in human glioblastoma, causing changes in the brain cells and vasculature. Clearly, by adding excessive expression of PDGF-B to the p53 null background we induced NSCs properties in more widespread areas of the brain, since neurosphere cultures could be established from the corpus callosum of PDGF-B/P53null brains, but not from the brains of PDGF-B or p53 null genotypes.

The fact that loss of p53 is necessary in brain tumor formation induced by PDGF-B in adult mouse brain has never been shown before, making this model different from previously described studies. However, in the PDGF-A\(\_\)transgenic model, presence of only one aberration was sufficient in the process of tumorigenesis, demonstrating a
crucial importance of increased growth factor activity in brain tumor formation. We found that PDGF-A_L could induce accumulation of immature OPC-like cells and astrocytic cells, in many different locations in the brain where the transgene was active, and in some cases overt neoplastic lesions. These results are in accordance with other studies in which expression of PDGF-A was inhibited in the brain, or in studies where PDGF-A_S was overexpressed, leading to a decreased or increased number of oligodendrocyte progenitors, respectively. However, one has to keep in mind that PDGF-A_L differs from PDGF-A_S by presence of the hydrophobic C-terminal tail.

One might expect more similarities between the two transgenic models, since PDGF-B and PDGF-A have quite a few features in common, like their proteolytic activation. Unlike PDGF-A, PDGF-B is only produced as a matrix-bound form (Heldin CH 1999b). Thus, both PDGF-B and PDGF-A_L share a high similarity between their respective retention motifs in the C-terminus. However, bound PDGF-B can be released from the cell surface by proteolytic activity (Field SL 1996). Conceivably, as freely diffusible in the extracellular fluid, PDGF-B can act at a distance from the producing cell, whereas the cell-surface attached PDGF-A_L is mostly affecting the cell that is producing it. PDGF-A and -B have different affinities for PDGF receptor binding and activation. PDGF-A can only induce PDGFR-α mediated signaling, while PDGF-B can induce PDGFR-α and -β receptor mediated signaling. Although, both receptors mediate very similar cellular responses, activation of PDGFR-β is very important, since NSCs and CSCs are known to depend on vascular niches. In our pre-tumor study with B/P53null mice we detected changes in the perivascular niche that could be crucial in allowing brain tumors to develop. However, infrequent glioma-like lesions develop also in PDGF-A_L overexpressing mice with increased vasculature, especially in the cerebellum. But the functional role of PDGF-A_L is poorly known and further studies will be required to elucidate the specific mechanisms by which overexpression of PDGF-A_L causes this phenotype.

In conclusion, the significance of this work is that by establishing two transgenic mouse models of glioma we could start to elucidate individual roles of two different members of the PDGF family. By comparing the phenotypic changes in the brains of these mice we will be able to further understand the key events in glioma development and thereby identify potential targets for therapy.
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