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PDGFB AND P53 IN BRAIN TUMORIGENESIS

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ABSTRACT

Glioblastoma is the most common, and malignant form of brain tumor. It is characterized by a rapid growth and diffuse spread to surrounding brain tissue. The cell of origin is still not known, but experimental data suggest an origin from a glial precursor or neural stem cell. Analysis of human glioma tissue has revealed many genetic aberrations, among which mutations and loss of *TP53* together with amplification and over-expression of *PDGFRA* are common. Many of the pathways that are found mutated in gliomas, are normally important in regulating stem cell functions.

We have investigated the role of p53 in adult neural stem cells, and found that the p53 protein is expressed in the SVZ in mice. Comparison of neurosphere cultures derived from *wt* and *Trp53-/-* mice showed that neural stem cells lacking p53 have an increased self-renewal capacity, proliferate faster and display reduced apoptosis. Gene expression profiling revealed differential expression of many genes, the most prominent being *Cdkn1a* (p21) which was down-regulated in *Trp53-/-* neural stem cells.

Mice lacking p53 do not develop gliomas, but the combination of *TP53* mutation/deletion together with other genetic aberrations is common in human gliomas of all grades. We generated a transgenic mouse model mimicking human glioblastoma, by over-expressing PDGFB under the *GFAP* promoter in *Trp53*$^{-/-}$ mice. The transgene was active in both neural stem cells and astrocytes. These mice developed malignant tumors resembling human glioblastoma at the age of 2-6 months. The tumors showed histopathological features of human glioblastoma, such as pseudopalisading necrosis, microvascular proliferation and pleomorphic nuclei. We used the same transgenic mouse model to study the brain before tumor formation. In the *PDGFB/Trp53-/-* brain we found increased numbers of Pdgf receptor alpha^{+} cells and prominent Pdgf receptor beta^{+} vessels in areas where brain tumor later developed. Neurosphere-forming cells were found in a more widespread location including corpus callosum. Thus, both the neural stem cells and the brain vasculature are affected by the combination of excessive PDGFB and loss of p53.

This investigation provides new insights into the roles of P53 and PDGF in brain tumor formation. We found that loss of p53 leads to deregulation of the stem cell compartment in the mouse SVZ. Expression of *PDGFB* in the NSCs and astrocytes of *Trp53-/-* brain, leads to the expansion of cells with neurosphere forming ability to other locations of the brain. As a result of the forced *PDGFB* expression in *Trp53-/-* brain, the vasculature is changed and eventually, high-grade gliomas develop.

LIST OF PUBLICATIONS

I. Meletis K, Wirta V, **Hede SM**, Nistér M, Lundeberg J, Frisén J.
p53 suppresses the self-renewal of adult neural stem cells
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GFAP promoter driven transgenic expression of PDGFB in the mouse brain leads to glioblastoma in a *Trp53 null* background
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III. **Hede SM**, Nazarenko I, He X, Eriksson A, Andrae J, Nistér M.
Stem cells and vessels in pretumorigenic mouse brain
Manuscript

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AML	Acute myelogenous leukaemia
AKT	V-akt murine thymoma viral oncogene homolog 1 /Protein kinase B
ARF	Alternative reading frame
bFGF	basic fibroblast growth factor
CDK	cyclin dependent kinase
CNS	central nervous system
EGF	epidermal growth factor
ERB	erythroblastic leukemia viral oncogene homolog /Her2
ES	embryonic stem (cell)
GBM	glioblastoma multiforme
GFAP	glial fibrillary acidic protein
HIF1a	hypoxia-inducible factor 1a
IDH1	isocitrate dehydrogenase 1
MAPK	mitogen-activated protein kinase
MET	hepatocyte growth factor receptor
MDM2	mouse double minute 2
MTOR	mammalian target of rapamycin
NG2	chondroitin sulfate proteoglycan 4
NSC	neural stem cell
OPC	oligodendrocyte progenitor cell
PDGF	platelet-derived growth factor
PTEN	phosphatase and tensin homolog
RB	retinoblastoma protein
RCAS	replication competent ALV splice receptor
SVZ	subventricular zone
TP53	tumor protein 53
WHO	world health organization

1 INTRODUCTION

1.1 BACKGROUND

New cells are constantly born in the human body, and also in the brain. The cell division process, by which one cell is divided into two, is strictly controlled in multiple ways. Still, sometimes a cell escapes all surveillance mechanisms and starts dividing without control, to eventually form a tumor.

The initiation of a tumor is a multi-step process. The tumor cells need to acquire several properties such as limitless replication potential, self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, sustained angiogenesis and metastatic ability (Hanahan and Weinberg 2000). These properties are gained by genetic or epigenetic changes that lead to subsequent inactivation of important cell regulatory functions.

The studies in this thesis concern the functional effects of genetic changes that lead to brain tumors in humans. Every year, about 1100 patients are diagnosed with brain tumor in Sweden, the majority of which are 60 years of age or older. The brain tumors constitute about 3 % of all cancers. The most common type of brain tumor, glioblastoma, is also the most malignant and lethal one. The median survival time after diagnosis is only 14 months, despite aggressive treatments with surgery, radiation and chemotherapy (van Meir et al. 2010).

A better understanding of the mechanisms behind brain tumor initiation and progression is needed to identify new potential targets for therapy of this deadly disease.

1.2 CLASSIFICATION OF HUMAN BRAIN TUMORS

Tumors found in the brain are either primary brain tumors originating from within the brain tissue, or metastasising tumors originating from a distant site. The most common primary brain tumors in the adult central nervous system (CNS) are gliomas. Gliomas are histopathologically classified as astrocytomas, oligodendrogliomas, mixed oligoastrocytomas and ependymomas. The gliomas are graded on a scale from I-IV, with grade IV being the most malignant. Pilocytic astrocytoma (WHO grade I) is a benign astrocytic tumor, rarely progressing to more malignant forms. Diffuse astrocytomas (WHO grade II) are slowly growing, diffusely infiltrating astrocytomas that are prone to progress to higher grades. Anaplastic astrocytomas (WHO grade III) display increased proliferation rate, increased cellularity and nuclear atypia. Glioblastoma multiforme (WHO grade IV) is the most malignant form of astrocytoma, characterized by microvascular proliferation, necrosis and cellular pleomorphism (Louis et al. 2007). The high-grade tumors can present *de novo* as primary glioblastomas, or evolve through progression from lower-grade tumors as secondary glioblastomas by gaining more mutations. The primary and secondary glioblastomas show the same histopathological features, although they differ in both genetic aberrations and the clinical history (Ohgaki and Kleihues 2007). The histopathological criteria for classification of astrocytomas are summarized in Table 1. The oligodendrogliomas are a separate entity of glioma believed to originate from oligodendrocyte progenitors, occuring as WHO grade II (oligodendroglioma) or WHO grade III (anaplastic oligodendroglioma).

WHO Grade	WHO Designation	Histological criteria
I	Pilocytic astrocytoma	
II	Diffuse Astrocytoma	Nuclear atypia
III	Anaplastic Astrocytoma	Nuclear atypia, mitotic activity
IV	Glioblastoma	Nuclear atypia, mitotic activity, vascular proliferation, necrosis

Table 1. Histopathological criteria for grading of astocytic tumors (Kleihues and Cavenee, 1997, Louis et al. 2007)

The difficulties of proper histopathological classification of gliomas have become more and more evident during the last years. Information on the classification and grading of brain tumors is essential for the choice of therapy. The histopathological classification also provides a predictive value for the clinical outcome of the patient. Recent results from genetic analyses of the tumors have suggested the existence of several subgroups within the histopathological classification system. A better understanding of the different glioma subtypes will result in better therapies for the individual patient as well as better stratification in clinical trials of new therapies for gliomas.

1.3 GENETIC AND EPIGENETIC CHANGES IN GLIOMAS

Molecular analyses of glial tumors have revaled a multitude of genetic and epigenetic aberrations present in the tumors. Although the glioma subtypes differ somewhat in their relative frequencies of genetic alterations, the changes commonly affect the major signaling pathways regulating cellular proliferation and survival.

1.3.1 Tyrosine kinase receptor signaling pathways

Excessive growth factor stimulation is common in gliomas. Stimulation of tumor cells is achieved by over-expression or genomic amplifications of both the secreted ligands and the receptors. The extracellular ligands, such as PDGF or EGF function by binding to protein tyrosine kinase receptors located in the outer membrane of the cell. The

ligand binding leads to receptor dimerization and transphosphorylation of the intracellular domains of the tyrosine kinase receptors. This in turn, allows for the activation of intracellular PI3K/AKT and RAS/MAPK signaling pathways regulating proliferation and survival of the cell. Eighty-eight % of glioblastomas have alterations in any of the components of the tyrosine kinase/RAS/PI3 kinase pathway (TCGA 2008).

EGF/EGFR: EGFR is a tyrosine kinase receptor that signals via the RAS and PI3 kinase pathways, stimulating cell division, survival and invasion. Amplifications of the EGFR gene are present in about 40% of glioblastomas, mostly of the primary type (Ekstrand et al. 1991; Ohgaki and Kleihues 2007). Constitutively activating genetic rearrangements are common in tumors with over-expressed *EGFR*. The most common *EGFR* mutant is the *EGFRvIII*, where a deletion of exons 2 to 7 results in a constutively active receptor variant without a functional ligand-binding domain. The EGFRvIII is present in 20-30% of all glioblastomas, and in about 50% of the tumors with *EGFR* amplification (Frederick et al. 2000; Sugawa et al. 1990).

PDGF/PDGFR: The PDGF ligands PDGF-A, -B, -C and –D are all expressed in glial tumors (Lokker et al. 2002). They bind to and activate tyrosine kinase receptors PDGFR-α on tumor cells and PDGFR-β on tumor vessels, to activate RAS and PI3 kinase signaling pathways. PDGFA and PDGFB as well as the PDGF receptors are expressed in gliomas (Nister et al. 1982; Nister et al. 1988). The expression patterns of PDGF ligands and receptors in glioma tissue suggest the presence of autocrine and paracrine loops stimulating tumor growth (Hermanson et al. 1992). *PDGFRA* over-expression is found in gliomas of all grades, and *PDGFRA* amplification is found in a subset of malignant gliomas (Fleming et al. 1992; Hermanson et al. 1996). Recent data show that about 13 % of glioblastomas have amplified *PDGFRA* (TCGA 2008).

Other tyrosine kinase receptors have also been shown to be altered in glioblastomas. *ERBB2* is mutated in 8% of glioblastomas, and the c-MET gene is amplified in 4% of glioblastomas (TCGA 2008).

RAS: Activated tyrosine kinase receptors activate RAS, which is a GTPase that stimulates both the MAPK and PI3K pathways. Although activating mutations of Ras are present in about half of all human tumors, glioblastomas rarely (2%) carry this

mutation (TCGA 2008). Active RAS is reqired for the proliferation of astrocytoma cells (Guha et al. 1997). Neurofibromin-1 (NF-1) is a protein that is mutated in the hereditary condition Neurofibromatosis type 1, and these patients have a predisposal for developing gliomas (Sorensen et al. 1986). NF-1 acts by inhibiting RAS activity, and *NF1* has recently been found to be mutated in 18% of glioblastomas (TCGA 2008).

PI3K: PI3 kinase is recruited to the tyrosine kinase receptors upon ligand binding and transphosphorylation. The PI3 kinase consists of a regulatory domain and a catalytic domain that converts phosphatidylinositol-4,5-bisphosphate (PIP2) to PIP3, that further activates downstream targets such as AKT. Activating mutations in the catalytic domain are commonly found in tumors, and mutated *PIK3CA* is found in 15% of glioblastomas (Samuels et al. 2004). Genetic aberrations affecting the genes coding for the regulatory domain are not common, although recent results claim that the *PIK3R1* gene is mutated in 10 % of glioblastomas (TCGA 2008).

PTEN: PTEN is an inhibitor of the AKT signaling pathway that is inactivated by mutation, deletion or by epigenetic mechanisms in as many as 50% of high-grade gliomas (Knobbe and Reifenberger 2003; Ohgaki et al. 2004). Inactivation of *PTEN* leads to hyperactivation of AKT and increased proliferation and migration. Mutations in *AKT* are infrequent, and amplifications are found in 2% of glioblastomas. AKT is a kinase that acts by phosphorylating target proteins to stimulate cell growth (through mTOR), survival (through inhibition of BAD) and proliferation (through inhibition of GSK3-β). AKT also potentiates HIF1-α, thereby promoting angiogenesis through expression of VEGF (Zundel et al. 2000).

1.3.2 P53 pathway

The P53 tumor suppressor induces cell cycle arrest, senescence or apoptosis in response to cellular stress such as DNA damage, oncogenic activation and hypoxia. Mutations or deletions of *TP53* are found in 50% of human cancers. In glioblastomas *TP53* mutations are found in 35 % of the cases (TCGA 2008). In addition to point mutations, loss of the chromosomal region 17p, containing *TP53*, is a common event in both low- and high-grade gliomas (Ohgaki et al. 2004; von Deimling et al. 1992). It was found

that 87% of glioblastomas have genetic alterations in any of the components of the P53 signaling pathway (TCGA 2008).

P53: P53 is a tetrameric protein that executes its effects by acting as a transcription factor, regulating thousands of genes. Under normal conditions, P53 is constantly produced with a high turnover rate as it is rapidly ubiquitylated by MDM2 and degraded by the proteasome. Upon cellular stress such as DNA damage, the P53 protein becomes stabilized by phosphorylation by kinases such as ATM, ATR or Chk2. The MDM2 protein that targets P53 for destruction by ubiquitylation is amplified in 10% of glioblastomas (Reifenberger et al. 1994). Other studies show that *MDM2* is over-expressed in as many as 50% of primary glioblastomas (Biernat et al. 1997). MDM4 suppresses the transcriptional activity of P53 and the gene is amplified in 4% of glioblastomas (Riemenschneider et al. 1999; Shvarts et al. 1996).

CDKN2A is a gene that encodes two different tumor suppressors: p16Ink4a and p14Arf (p19Arf in mouse). This gene locus is deleted in 40-50% of glioblastomas. P14Arf acts by sequestering MDM2, and thereby increasing P53 levels in the cell. Loss of p14Arf expression by homozygous deletion of by promoter methylation is found in as many as 76% of glioblastomas (Nakamura et al. 2001).

1.3.3 RB pathway

The RB pathway regulates progression of the cell cycle at the transition from G1 to S-phase. The RB protein functions by binding to and inhibiting the E2F transcription factors. Phosphorylation of the RB protein leads to release of E2F factors and progression to S-phase. Genetic alterations affecting genes involved in the RB pathway were found in 78% of glioblastomas (TCGA 2008). The *RB1* gene is commonly mutated in humans cancers, and in glioblastomas it was found mutated or deleted in 11% of the tumors (TCGA 2008). Promoter methylation and silencing of *RB1* is more commonly found, in 43% of secondary gliomas and in 14% of primary glioblastomas (Nakamura et al. 2001). The protein kinases that regulate RB function are often found amplified in glioblastomas. Amplification of *CDK4* for example, occurs in 14% of glioblastomas. Further upstream in the RB signaling pathway, the *CDKN2A*, *CDKN2B* and *CDKN2C* genes are commonly found mutated (in 52, 47 and 2%, respectively).

They encode CDK inhibitors p16Ink4a, p15Ink4b and p18Ink4c, that bind to and inhibit CDK4/6-CyclinD complexes.

Abrogation or dysregulation of any of the cell cycle regulatory pathways P53 or RB alone is not enough for induction of glial tumors, but makes the cell more susceptible to transformation by mitogenic signaling through PI3K and MAPK pathways (Furnari et al. 2007).

1.4 GENETIC CLASSIFICATION OF GLIOMA SUBTYPES

Recent advances in gene sequencing, gene expression profiling and proteome analysis of glioma tumors have provided insights into the mechanisms of glioma development and progression as well as identified new subgroups (Brennan et al. 2009; Phillips et al. 2006; TCGA 2008).

Primary glioblastomas are subdivided into four separate groups, based on their gene expression profiles (Figure 1):

- The classical primary glioblastomas are characterized by *EGFR* amplification and loss of *PTEN* and *CDKN2A*. Mutations in *TP53* are rarely found in this group of tumors. Activation of Notch as well as Shh pathways are common.
- The mesenchymal primary glioblastomas are characterized by mutation or loss of *NF1*, *TP53* and *PTEN*. They have a mesenchymal or angiogenic gene expression profile with activated TNF and NFκB signaling pathways.
- The proneural primary glioblastomas are characterized by *PDGFRA* amplification, loss or mutations of *TP53, CDKN2A* and *PTEN*. Tumors of the proneural subtype also often show mutations in *PIK3CA/PI3R1* and *IDH1*. They have a gene expression profile resembling that of neuronal and oligodendrocyte progenitor cells. The characteristics of the proneural subtype are very similar to secondary glioblastomas.
- The neural primary glioblastomas are characterized by *EGFR* amplification or overexpression, and a gene expression profile of normal brain. This subtype is less defined than the other types.

In addition to alterations of the cell cycle regulatory pathways, other genetic events are important in modulating for example tumor progression and invasion. Recent efforts in global sequencing of glioblastoma tumors have resulted in the identification of genetic changes earlier unknown in gliomas. One example is isocitrate dehydrogenase 1 (IDH1) and IDH2 mutations. These proteins have metabolic functions, and were found mutated in both low-grade and high-grade tumors, especially in secondary glioblastomas (Parsons et al. 2008; Yan et al. 2009).

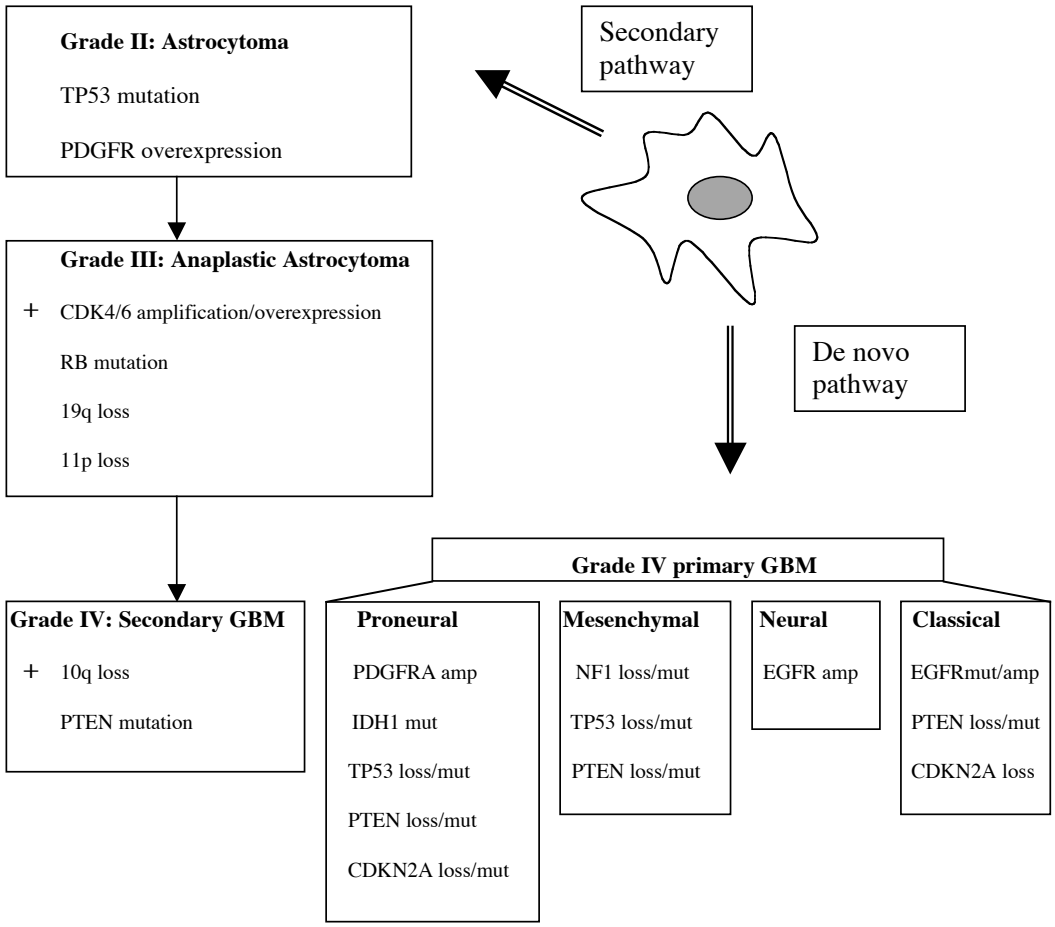

Figure 1. Common genetic aberrations in primary and secondary glioblastoma (GBM) (Adapted from van Meir et al. 2010).

1.5 PDGF

1.5.1 PDGF ligands and receptors

The platelet-derived growth factor (PDGF) proteins are members of the evolutionary conserved VEGF/PDGF family of proteins. They are all disulfide-linked polypeptide chains, with a core domain containing eight conserved cysteine residues (McDonald and Hendrickson 1993). The PDGF family of growth factors includes five different dimeric proteins: PDGF-AA, -AB, -BB, -CC, -DD. Four genes *PDGFA*, *PDGFB*, *PDGFC* and *PDGFD* encode the polypeptides that form these PDGF ligands. The expression patterns of the different PDGF genes are generally non-overlapping, and the PDGF-AB heterodimers are rarely found *in vivo* (Hoch and Soriano 2003). The PDGF polypeptide chains PDGF-A and PDGF-B are produced as pro-proteins, which are proteolytically cleaved inside the cell after dimer formation. Both PDGF-B and PDGF-A polypeptides contain C-terminal retention motifs that mediate binding to extracellular matrix (ECM) components (LaRochelle et al. 1991). PDGF-A has two splice variants; the longer variant PDGF-A_L, with the retention motif that provides ECM binding properties, and a shorter splice variant PDGF-A_S, that is freely diffusible (Pollock and Richardson 1992). The PDGF-C and PDGF-D polypeptides on the other hand contain an N-terminal inactivating CUB-domain. This domain sterically prevents receptor binding until cleavage and activation by extracellular proteases (Bergsten et al. 2001) (Li et al. 2000).

The PDGF proteins bind to and activate PDGF receptors alpha and beta located in the cell membrane. Upon ligand binding the tyrosine kinase receptors form homo- or heterodimers. Each PDGF receptor dimer consists of two polypeptide chains, each with an extracellular immunoglobulin-like ligand-binding domain, a transmembrane domain and a split intracellular tyrosine kinase domain. Ligand binding and dimerization leads to transphosphorylation of the intracellular domains and subsequent activation of intracellular signaling pathways via recruitment of several intracellular substrates to the intracellular receptor domains. The PDGF receptors and the PDGF ligand binding specificities are summarized in Figure 2.

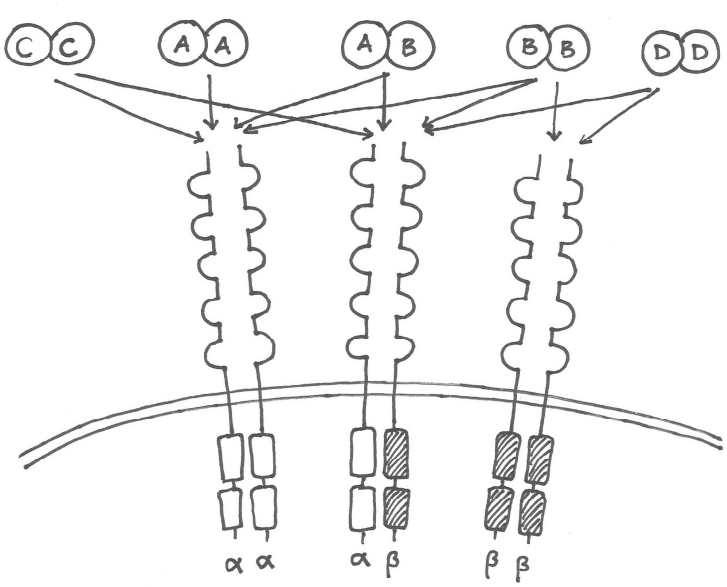

Figure 2. PDGF ligands and their interaction with the PDGF receptors

1.5.2 PDGF functions during development

The PDGF ligands and receptors have important functions both during development and in the adult. Originally, PDGF was identified as a potent mitogenic factor for smooth muscle cells (Ross et al. 1974), fibroblasts (Brewitt and Clark 1988) and glial cells (Westermark and Wasteson 1976). The PDGF ligands and receptors are important in developmental processes during embryogenesis. The developmental roles of PDGF signaling have been extensively studied in gene-targeted mouse models, where knock-out of any of the genes encoding PDGF ligands and receptors results in severe developmental defects (Bostrom et al. 1996; Lindahl et al. 1997). The somewhat overlapping phenotypes of the different PDGF ligand and receptor knock-out mice, have led to the conclusion that PDGF-AA and PDGF-CC act via the PDGF receptor alpha, while PDGF-BB mainly signals via PDGF receptor beta *in vivo* (Ding et al. 2004; Soriano 1994).

PDGF-A is important for the development of alveoli in the lung, intestinal villi, hair follicles and in spermatogenesis. PDGF-C plays important roles in for example palate formation. PDGF-B stimulates pericytes and vSMCs in angiogenesis and is also involved in the formation of glomeruli of the kidney. The developmental functions of PDGF-D is still very much unknown. Disturbed PDGF signaling is implicated in many

different pathological processes such as in wound healing, atherosclerosis, fibrosis and tumorigenesis (reviewed by Andrae et al. 2008).

1.5.3 PDGF functions in brain

During development of the CNS and in the adult brain, PDGF-A is expressed by astrocytes and neurons (Fruttiger et al. 2000; Noble et al. 1988; Yeh et al. 1991). PDGF-A signaling through PDGF receptor alpha mediates glial progenitor cell survival, proliferation and migration (Armstrong et al. 1990; Calver et al. 1998). The amount of PDGF-A produced seems to be rate limiting for the number of oligodendrocyte progenitors produced both in the embryo and in the adult brain (van Heyningen et al. 2001; Woodruff et al. 2004). The PDGF receptor alpha is expressed both in neural stem cells, oligodendrocyte progenitors, astrocytes and neurons (Doetsch et al. 1999; Hart et al. 1989; Pringle and Richardson 1993; Schatteman et al. 1992). Meninges and choroid plexus also express PDGF receptor alpha (Pringle et al. 1992).

PDGF-A acts as a mitogenic factor and influences differentiation of glial progenitors (Barres et al. 1992). Experiments *in vitro*, have shown that PDGF-A can induce embryonic $Nestin^{+}$ neural progenitor cells into becoming $NG2^{+}$ oligodendrocyte precursors (Hu et al. 2008). In the adult SVZ, stimulation with PDGF-A induces PDGF receptor alpha expressing NSCs to give rise to oligodendrocytic lineage cells instead of neuronal lineage cells (Jackson et al. 2006; Menn et al. 2006).

PDGF-B is expressed in both embryonal and adult neurons (Sasahara et al. 1991). Neurons also express PDGF receptor beta, which mediates a neuroprotective function after injury (Smits et al. 1991) (Egawa-Tsuzuki et al. 2004) (Ishii et al. 2006). PDGF receptor beta is also found on fibroblasts and in the brain vasculature, mainly on pericytes (Hellstrom et al. 1999) (Smits et al. 1989). In addition to PDGFR beta, both fibroblasts and smooth muscle cells express PDGFR alpha, although at lower levels (reviewed by Heldin and Westermark 1999).

1.6 P53

P53 is a protein that upon tetramerisation acts as a transcription factor, repressing or enhancing the transcription of hundreds of target genes with P53 response elements (el-Deiry et al. 1992). Indirectly, P53 regulates the activity of thousands of genes in the cell (Wang et al. 2001). Under normal conditions the level of P53 protein is held at a steady state as P53 is constantly produced with a rapid turnover rate. MDM2 is the main regulator of P53 levels in the cell, as it ubiquitinates P53 and targets it for destruction by the proteasome.

P53 controls numerous cellular and developmental processes, such as apoptosis, cell-cycle arrest, DNA repair, metabolism, senescence, differentation, autophagy and inhibition of angiogenesis. It has an important role in preventing tumor formation, and is crucial for maintaining genome stability (Lane 1992). The tumor suppressor gene *TP53* is mutated or lost in about 50% of human tumors (Hollstein et al. 1991). The rest often have inactivated the P53 regulatory pathway by other mechanisms, such as by loss of *ARF* or over-expression of *MDM2* (Soussi and Wiman 2007).

The most important function of P53 is to sense and to control the cellular response to different intrinsic or extrinsic stress signals. DNA damage, oncogene signaling, hypoxia, ribosomal stress or metabolic stress signals lead to accumulation and stabilization of the P53 protein, and a subsequent cellular response (Ashcroft et al. 2002). There are several alternative outcomes for the cell after P53 activation. The cellular response to P53 activation depends on the type of tissue, nature of stress signal, cellular environment, extent of damage and duration of stress (Chen et al. 1996).

Activated P53 stops cell cycle progression at the G1/S checkpoint transiently, by increased transcription of the CDK inhibitor $p21^{Cip1}$ *(CDKN1A)*. A terminal blockade of cell cycle progression by senescence can be induced by p21 together with other factors such as PAI-1 (plasminogen activator inhibitor 1). Increased levels of P53 can also induce cell death by apoptosis, mainly by transcriptional activation of *PUMA* (p53-upregulated modulator of apoptosis). Increased levels of P53 also induce DNA repair proteins. In case of lack of nutrients or oxygen, P53 can alter the metabolism of the cell,

by for example inhibition of mTOR or altering the glucose uptake (Vousden and Prives 2009).

1.7 ANIMAL MODELS FOR BRAIN TUMORS

Rodents, and especially mice are commonly used to model brain tumors. Several methods are employed to mimic brain tumors in mice. Introduction of the genetic aberrations found in human brain tumors provides a system to study the mechanisms of brain tumor initiation and progression as well as to find new targets for treatment and subsequently test new therapies.

Different approaches are used to model human brain tumors in mice. In xenograft models, brain tumor cells are grown in vitro and subsequently transplanted into the brain of mostly immunocompromised mice. Somatic cells are targeted by retro-, adeno- or lentiviral vectors to express oncogenes. Transgenic mice with germ-line modifications are created by pro-nuclear injection of DNA into fertilized mouse eggs. Germ line deletions of genes, to create "knock-out" mice, are generated by homologous recombination in embryonic stem (ES) cells. The use of tissue-specific and inducible promoters can direct over-expression of oncogenes or deletion of tumor suppressor genes to a specific cell type, at a specific timepoint. Increasingly sophisticated methods to target specific cells at a given time point together with advances in fluorescence labeling and in vivo imaging techniques are evolving rapidly.

1.7.1 Experimental gliomas induced by excessive PDGF signaling

Glioma-like tumors have been induced by PDGF in different mouse model systems. Excessive PDGF ligand causes expansion of oligodendrocyte precursors and leads mainly to oligodendroglial tumors in both newborn and adult mouse brain.

Retroviral expression of PDGFB in embryonic neural progenitors induces highly malignant oligodendroglial tumors in mice (Appolloni et al. 2009; Calzolari et al. 2008). Experiments in newborn rats show that injection of retroviral PDGFB can lead to a shift in the differentiation fate of the NSCs, producing more Pdgfra/NG2/Olig2

expressing oligodendrocyte progenitor cells (OPCs). In addition, the OPCs halt in their maturation and do not differentiate into mature oligodendrocytes (Assanah et al. 2009). Retroviral over-expression of PDGFB in the newborn mouse brain on the other hand induces oligodendrogliomas (Uhrbom et al. 1998).

A more restricted approach, using the RCAS-TVA system to direct PDGF over-expression to Gfap, Nestin or CNPase expressing cells in the newborn mouse brain, also results in oligodendroglial tumors. In this model some Gfap-tva and CNPase-tva mice also developed mixed oligo-astrocytomas (Dai et al. 2001) (Lindberg et al. 2009). These PDGFB-induced brain tumors are often altered to a more malignant type when combined with a second genetic aberration such as loss of p53, Ink4a/Arf or $p27^{Kip1}$ (Hesselager et al. 2003; See et al. 2010; Tchougounova et al. 2007). The histopathological features of the PDGFB-induced oligodendrogliomas produced using the RCAS-TVA system are also dependent on the amount of PDGFB. Increasing doses of PDGFB has been shown to increase tumor cellularity and angiogenesis by recruitment of vSMCs (Shih et al. 2004).

Retroviral expression of PDGFB in the adult rat subcortical white matter (corpus callosum) lead to the infection and transformation of NG2+ oligodendrocyte progenitor cells and development of malignant glioblastomas (Assanah et al. 2006). Gliomas can also be induced by forced PDGFB expression in Gfap expressing cells of the spinal chord in mice (Hitoshi et al. 2008). Over-expression of PDGFB in oligodendrocytes, using the myelin basic protein (MBP) promoter leads to an increase of oligodendrocyte precursors in the adult mouse brain (Forsberg-Nilsson et al. 2003). In the adult mouse brain, intraventricular perfusion with PDGF-A leads to reversible glioma-like lesions constituted by Pdgfra+, Nestin+ and Olig2+ tumor cells (Jackson et al. 2006).

1.7.2 Experimental gliomas by inactivation of the P53 pathway

Genetic mouse models for glioma show that inactivation of the p53 pathway, needs to be combined with loss of function of a second regulatory pathway for brain tumors to develop. Mice deficient in p53 have a short life-span, as they develop several tumor types, mostly lymphomas and sarcomas within approximately six months after birth (Donehower et al. 1992). Targeted deletions of *Trp53* in brain tissue have been used to study the brain tumorigenic role of p53 together with other genetic aberration. Different

strategies and combinations of targeted genes and targeted cell types has given valuable clues about the brain tumor initiating events.

Several mouse model systems have targeted genetic aberrations to specific cell types of the brain. Deletion of *Trp53* in all Gfap expressing cells using the Cre-loxP system, does not result in gliomas (Marino et al. 2000). However, the combined loss of p53 and other cell cycle regulating factors can give rise to glial tumors. For a high grade glial tumor to develop, inactivation of the p53 regulatory pathway needs to be combined with loss of function of a second cell cycle controlling mechanism, such as the Rb pathway, or combined with overactivation of growth factor signaling such as the Ras/MAPK or PI3K/AKT pathways (Furnari et al. 2007).

1.7.3 Deregulation of P53 and growth factor signaling pathways

Astrocytomas (grade II-IV) have been induced in mice by genetic disruption of *Trp53* together with loss of *Nf1* (Reilly et al. 2000). A more restricted approach where *Trp53* and *Nf1* were deleted, or mutated, in only Gfap expressing cells resulted in a similar tumor phenotype with high-grade astrocytomas (Wang et al. 2009; Zhu et al. 2005). These high-grade tumors were only found when *Trp53* was deleted before or at the same time as *Nf1*, indicating that loss of *Trp53* was crucial for tumor initiation. Loss of *Nf1* before loss of *Trp53*, on the other hand rarely resulted in brain tumors (Zhu et al. 2005). The addition of somatic heterozygosity of *Pten* to loss of *Trp53* and *Nf1* in Gfap expressing cells results in shortening of tumor latency and tumor progression (Kwon et al. 2008). Deletion of *Trp53, Nf1* and *Pten* in both embryonic and adult Nestin-expressing progenitor cells also results in high-grade astrocytomas (Alcantara Llaguno et al. 2009).

The combination of targeted deletions of *Trp53* and *Pten* in Gfap expressing cells, lead to grade III-IV astrocytic tumors (Zheng et al. 2008). Deletion of *Trp53* and *Pten* using an adenovirally expressed Gfap-Cre restricts the targeted cells to only Gfap expressing cells of the SVZ, and results in anaplastic astrocytomas (grade III). In the same experimental set up, the combined deletion of *Rb* together with *Trp53* or the combined deletion of *Rb* and *Trp53* and *Pten* give rise to primitive neuroectodermal tumors (PNETs) (Jacques et al. 2010). Loss of Rb function has also been achieved by transgenic expression of the SV40 antigen T_{121} in Gfap expressing cells. These mice

develop grade III astrocytomas, and the tumor progression is accelerated in *Pten* heterozygous, but not in *Trp53* heterozygous mice (Xiao et al. 2002).

The experimental results on the brain tumorigenic capacity of activated Ras in animal models are contradictory. In one model, transgenic expression of constitutively active Ras in Gfap expressing cells induces both low- and high-grade multifocal astrocytic tumors, in a dose-dependent manner (Ding et al. 2001). In another inducible model system, activation of K-ras in adult Gfap expressing cells only resulted in grade II-III astrocytic tumors (Abel et al. 2009). Results from other models systems show that activation of Ras alone is incapable of inducing gliomas (Holland et al. 2000; Marumoto et al. 2009).

Somatic gene transfer of the combination of K-Ras and Akt into newborn mice by using the RCAS-tva system results in high-grade astrocytomas when targeting Nestin expressing progenitors, but no tumors develop when targeting Gfap expressing cells (Holland et al. 2000). In adult brain, lentiviral vectors with H-Ras and Akt can induce grade III astrocytomas when introduced in Gfap expressing cells of SVZ as well as hippocampus. When introduced in *Trp53* heterozygous mice, on the other hand, more malignant glioblastoma-like tumors developed in Gfap expressing cells of SVZ, hippocampus and in some cases cortex (Marumoto et al. 2009).

Loss of Arf, a critical component regulating p53, can also cause glioma formation in mice when combined with an overactive growth factor signaling pathway. Retroviral expression of constitutively activated EGFR in both Ink4a/Arf null astrocytes and neural stem cells lead to high-grade gliomas (Bachoo et al. 2002). Deletion of the Ink4a/Arf locus together with oncogenic Ras introduced via the RCAS-tva system into Gfap or Nestin expressing cells of newborn mice, results in glioblastoma-like brain tumors (Uhrbom et al. 2002).

Loss of *Trp53* together with loss of *Pten* was also found to induce high-grade gliomas (Zheng et al. 2008). The combined deletion of *Trp53* and *Pten* leads to gliomas in Gfap expressing neural stem cells of the SVZ, but not in Gfap expressing differentiated cells (Jacques et al. 2010).

In summary, the work in animal model systems show that inactivation of any two of the P53, RAS or PI3K pathways can induce glial tumors. Inactivating a third pathway will induce a more malignant phenotype. They also show that different combinations of genetic alterations can lead to gliomas with a very similar phenotype. All glioblastoma models targeting the SVZ neural stem cells specifically require loss of the p53 pathway.

The techniques of targeting individual cell types in specific regions of the have evolved quickly, from knock-out mice with all cells in the entire organism targeted by the genetic lesion, to lentiviral gene transfer where only a few cells in a specific region are transduced at a specific time point. The use of targeted approaches to hit a more restricted cell population will allow for a more accurate interpretation of the processes necessary for brain tumor initiation.

1.8 NEURAL STEM CELLS

During embryonic development, what started out as a single totipotent cell soon develops into a pluripotent blastocyst. As the stem cells of the embryo differentiate to form organs and tissues, potency is gradually restricted. Multipotent stem cells give rise to all the cell types within a tissue, and are often residing in specialized niches of adult tissues. These tissue stem cells have the ability to self-renew through both symmetric and asymmetric cell divisions. The tissue stem cells are often quiescent, non-dividing cells that upon reactivation give rise to rapidly dividing progenitor cells. By asymmetric cell division the stem cell pool is maintained at the same time as differentiated progeny is produced.

The neural stem cells (NSCs), are found in two areas of adult mammalian brain; the subventricular zone (SVZ) of lateral ventricular wall and the dentate gyrus in hippocampus. NSCs have the capacity to give rise to cells of astrocytic, oligodendrocytic and neuronal cell lineages.

The discovery of NSCs in the adult brain was made only recently. For a long time, neurogenesis was not considered to occur in the adult mammalian brain. In the 1960s came the first evidence of adult neurogenesis in rat brain (Altman 1962). Neural stem

cells have since then been identified in several species, including human (Doetsch et al. 1999; Kukekov et al. 1999). Neural stem cells can be isolated and cultured in vitro as neurospheres under serum-free conditions with the addition of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (Reynolds and Weiss 1992). Human neural stem cells have also been isolated by selection for stem cell specific cell surface markers, such as CD133 (Uchida et al. 2000).

The richest source of neural stem cells in the adult mammalian brain is the SVZ of the lateral ventricular wall (Doetsch and Alvarez-Buylla 1996). The lateral ventricles are lined by a monolayer of ciliated ependymal cells. Underneath the ependymal layer is the SVZ, where the neural stem cells reside. Neural (also called type B-cells) give rise to transit amplifying cells (TA or type C cells), which in turn give rise to both oligodendrocyte progenitors and neuroblasts (type A cells) (Doetsch et al. 1997; Nait-Oumesmar et al. 1999). The type B cells are slowly proliferating, while the transit amplifying progenitors are rapidly dividing to give rise to more differentiated progeny (Doetsch et al. 1997; Morshead et al. 1994).

The composition of the SVZ is somewhat different between mouse and human. The mouse SVZ lacks a hypocellular gap, which is found between the ependyme and astrocytic cells in the human SVZ. The neuroblasts of mouse SVZ form migratory chains, something that is not seen in humans (Lois et al. 1996; Quinones-Hinojosa et al. 2006). The human SVZ also generally contains fewer proliferating cells than mouse SVZ (Quinones-Hinojosa et al. 2006; Sanai et al. 2004).

Detailed analysis of the ventricular surface in adult mice show that both NSCs and ependymal cells have cilia. The apical end of the type B neural stem cell is located at the ventricular lining among the ependymal cells, and its other end contacts blood vessels in the SVZ (Mirzadeh et al. 2008). The type B neural stem cells are characterized by expression of both Gfap and Nestin (Doetsch et al. 1999; Lendahl et al. 1990). The B1 neural stem cells are derived from radial glial cells during development (Merkle et al. 2004).

1.9 NEURAL STEM CELLS AND TUMOR CELLS SHARE THE SAME REGULATORY PATHWAYS

The pathways regulating neural stem cell growth and differentiation are often the same as are found deregulated in brain tumors.

EGF and PDGF signaling pathways are often found over-activated in human gliomas of all grades. Both EGF and PDGF stimulate proliferation of neural stem cells in vitro. PDGF has a dedifferentiating effect on mouse astrocytes in vitro, evidenced by the induction of a gene expression pattern of glial precursors (Dai et al. 2001). In vivo, infusion of PDGF-A into adult mouse ventricles will induce the proliferation of Pdgf receptor alpha-expressing neural stem cells in the SVZ, and ultimately glioma-like reversible lesions (Jackson et al. 2006).

EGFR is expressed on transit amplifying progenitor cells of the SVZ, and EGF is used to keep NSCs in an undifferentiated state in neurosphere cultures in vitro (Reynolds and Weiss 1992). Retroviral introduction of constitutively active EGF receptor induces dedifferentiation of Ink4a/Arf-deficient mouse astrocytes in vitro. Both the dedifferentiated astrocytes and neural stem cells carrying the same genetic alterations were able to form high-grade gliomas in vivo (Bachoo et al. 2002).

PTEN is mutated or inactivated by epigenetic mechanisms in about half of all high-grade gliomas (Knobbe and Reifenberger 2003). PTEN functions as an inhibitor of the AKT pathway, and loss of PTEN leads to hyperactivation of AKT (Stambolic et al. 1998). Loss of Pten in Nestin expressing progenitor cells leads to an abnormal brain development, with an enlarged brain (Groszer et al. 2001). Studies on *Pten null* neurosphere cultures show that loss of Pten in neural stem cells leads to an increased self-renewal by promoting exit from the quiescent G_0 state to enter the cell cycle (Groszer et al. 2006). *PTEN* is also a transcriptional target of activated P53 (Stambolic et al. 2001).

The tumor suppressor gene *TP53* is often found inactivated in low-grade gliomas, indicating that loss of P53 is an early event in brain tumorigenesis. P53 is well known to induce apoptosis of neurons and neural progenitors during development (D´Sa-Eipper et al. 2001). An aberrant neuronal development and exencephaly during

embryonal development has been reported in *Trp53 null* mice (Armstrong et al. 1995; Sah et al. 1995). Adult neurons can undergo apoptosis by activation of P53 after various insults, such as irradiation, chemotherapeutic agents and ischemia.

In the mouse brain, p53 is expressed in the SVZ, regulating both proliferation, apoptosis and the self-renewing capacity of NSCs. Loss of p53 leads to an increased proliferation rate as well as decreased apoptosis. Analysis of gene expression profiles revealed down-regulation of the cell cycle regulatory factors p21 and p27 in *Trp53null* neural stem cells (Meletis et al. 2006). P21 is an important regulator of cell cycle progression and maintenance of quiescence in neural stem cells. Targeted deletion of *Cdkn1a* (p21) results in an initial expansion of the neural progenitor cell pool, followed by depletion of the neural stem cells (Kippin et al. 2005).

P53 has also been reported to regulate neural stem cell differentiation capacity, as neurospheres derived from both SVZ and olfactory bulb of *Trp53* knock-out mice are more prone to differentiate into the neuronal (Tuj1+) lineage (Armesilla-Diaz et al. 2009; Gil-Perotin et al. 2006). Loss of *Trp53* was reported to induce hyperplastic lesions in SVZ, which also display an increased susceptibility to mutagenic agents (Gil-Perotin et al. 2006). The genomic stability of the neural stem cells is also affected by loss of p53. Neurosphere cultures derived from the olfactory bulb of *Trp53* knock-out mice were shown to display an increased genomic instability with aneuploidy and chromosomal rearrangements (Armesilla-Diaz et al. 2009).

Olig2 is a transcription factor that is expressed in all types of glial tumors, irrespective of grade. Olig2 is expressed in, and necessary for the development of both oligodendrocyte progenitor cells and oligodendrocytes (Ligon et al. 2004; Ligon et al. 2006). In the neural stem cell compartment of adult SVZ, Olig2 expression is found in the type C transit amplifying cells (Hack et al. 2005). Olig2 suppresses the expression of p21 in neural stem cells (Ligon et al. 2007). Another stem cell-regulating factor often expressed in human gliomas is Bmi-1. Neurospheres lacking Bmi-1 have elevated levels of p21 (Molofsky et al. 2003).

1.10 CANCER STEM CELLS

1.10.1 Cancer stem cell theory

Lately, a new concept has emerged in the tumor biology field. The cancer stem cell theory suggests the existence of a small subpopulation of cancer cells with the capacity of unlimited self-renewal, thereby maintaining the tumor and giving rise to all of the differentiated tumor cells that constitute the bulk of the tumor (Reya et al. 2001).

The first evidence of a cancer stem cell population came from studies of acute myelogenous leukemia (AML) (Bonnet and Dick 1997). Since then cancer stem cells have also been identified in many solid tumors such as breast (Al-Hajj et al. 2003), brain (Singh et al. 2004), pancreas (Hermann et al. 2007), prostate (Miki et al. 2007), colon (O'Brien et al. 2007), and liver cancer (Alison et al. 2009) and in melanoma (Schatton et al. 2008) and teratoid/rhabdoid tumors (Chiou et al. 2008).

The term cancer stem cell refers to the self-renewal capacity and the ability to produce differentiated progeny. These stem cell properties may either be the result of mutations in a stem cell, or tumorigenic events leading to de-differentiation of a mature cell and gain of stem cell features. The nomenclature is a matter of confusion, and the terms tumor propagating cell or tumor initiating cell are also used to describe cancer stem cells (Clarke et al. 2006). Alternatively, the term tumor initiating cell is also used to describe the tumor cell of origin, early in the transformation process (Reilly et al. 2008).

The standard assays to functionally evaluate cancer stem cells assess the ability to propagate serially in an undifferentiated state *in vitro*, and to form phenotypically similar tumors by orthotopic transplantation (Reilly et al. 2008)

1.10.2 Cancer stem cells in glioma

Clonogenic neurosphere cultures were first established from human cortical glioblastoma samples (Ignatova et al. 2002). Brain tumor stem cells have since then been isolated from many types of human brain tumors, including both glioblastomas

(Galli et al. 2004; Singh et al. 2004), medulloblastomas (Hemmati et al. 2003) and ependymomas (Taylor et al. 2005). It was also found that orthotopic transplantation of brain tumor-derived cancer stem cells into mouse brain, resulted in tumors recapitulating the phenotype of the human brain tumor (Galli et al. 2004).

The selection for cancer cells displaying specific markers, such as CD133, can be used to enrich for the brain tumor stem cells (Singh et al. 2003). Transplantation experiments initially showed that CD133+ brain tumor cells were highly tumorigenic in comparison to CD133- brain tumor cells (Singh et al. 2004). Later reports show that also CD133- brain tumor cells form tumors in orthotopic transplant experiments (Beier et al. 2007). Studies on human glioblastomas show that equally tumorigenic clones of CD133+ and CD133- cancer cells co-exist within the same tumor, and that the CD133 expression is not static or lineage-restricted (Chen et al. 2010). CD133 is not specifically expressed in brain tumor stem cells, or even in neural stem cells. It is a marker of many different cell types, such as endothelial precursor cells, radial glia and ependymal cells (Pfenninger et al. 2007). Many other markers for neural stem- or progenitor cells are found expressed in brain tumor stem cells. These include Musashi, Bmi-1, Sox2 (Hemmati 2003), Nestin and Notch proteins (Fan et al. 2006).

1.10.3 Cancer stem cell niches

Stem cells are located in specialized niches, which provide a supportive and nurturing environment (Doetsch 2003; Fuchs et al. 2004). Both SVZ and hippocampal NSCs are found in close proximity to blood vessels (Louissaint et al. 2002; Palmer et al. 2000). The endothelial cells of blood vessels release factors that are promoting the self-renewal of stem cells (Ramirez-Castillejo et al. 2006; Shen et al. 2004). The NSC niche of the SVZ consists of many components such as ependymal cells, basal lamina, axonal projections and blood vessels (Riquelme et al. 2008). The brain is shielded from the blood stream by the blood brain barrier (BBB) that is composed of astrocyte endfeet closely connected with endothelial cells (Abbott et al. 2006). However, in the SVZ, both neural stem cells and transit amplifying cells are in close contact with capillary blood vessels lacking both astrocyte endfeet and pericytes. This is unique to the SVZ neural stem cell niche and not seen in other areas of the brain (Tavazoie et al. 2008).

During the development of brain tumors, angiogenesis is an important step in tumor progression, and high-grade gliomas are characterized by microvascular proliferations. Glioblastoma cells are also often found to migrate and spread into surrounding tissue along blood vessels (Sherer et al. 1940). Cancer stem cells have been identified in perivascular niches of human glioblastoma, ependymoma, oligodendroglioma and medulloblastoma. In these tumors, Nestin and CD133 expressing tumor cells were located near microvessels in close connection with endothelial cells, and endothelial cells secrete factors that promote self-renewal and proliferation of the brain tumor stem cells (Calabrese et al. 2007; Folkins et al. 2007). CD133 expressing tumor cells also express high levels of the pro-angiogenic factor vascular endothelial growth factor (VEGF), which also contributes to an elevated tumor-initiating capacity (Bao et al. 2006).

1.11 INTERACTIONS IN TUMOR TISSUE

Tumor cells are located in a heterogeneous microenvironment composed of several different cell types and extracellular matrix. The tumor cells interact with the surroundings via cell-cell interactions, cell-matrix interactions and by paracrine interactions (Pietras and Ostman). The brain tumor mass contains both differentiated tumor cells and cancer stem cells. Reactive astrocytes and microglia are also found, along with tumor stroma including endothelial cells and pericytes (Figure 3). There are now many examples where the tumor microenvironment and stromal cells affect tumor progression (Anderberg et al. 2009).

Glioblastomas are highly heterogeneous tumors (Noble and Dietrich 2004). The brain tumor microenvironment is very diverse and variable as one site can be hypoxic while other areas are highly vascularized. Different subclones of tumor cells display different gene expression patterns as well as different sets of mutations (Ren et al. 2007). Recent findings suggest that the intratumoral heterogeneity in gliomas may even be driving the tumor growth (Inda et al. 2010).

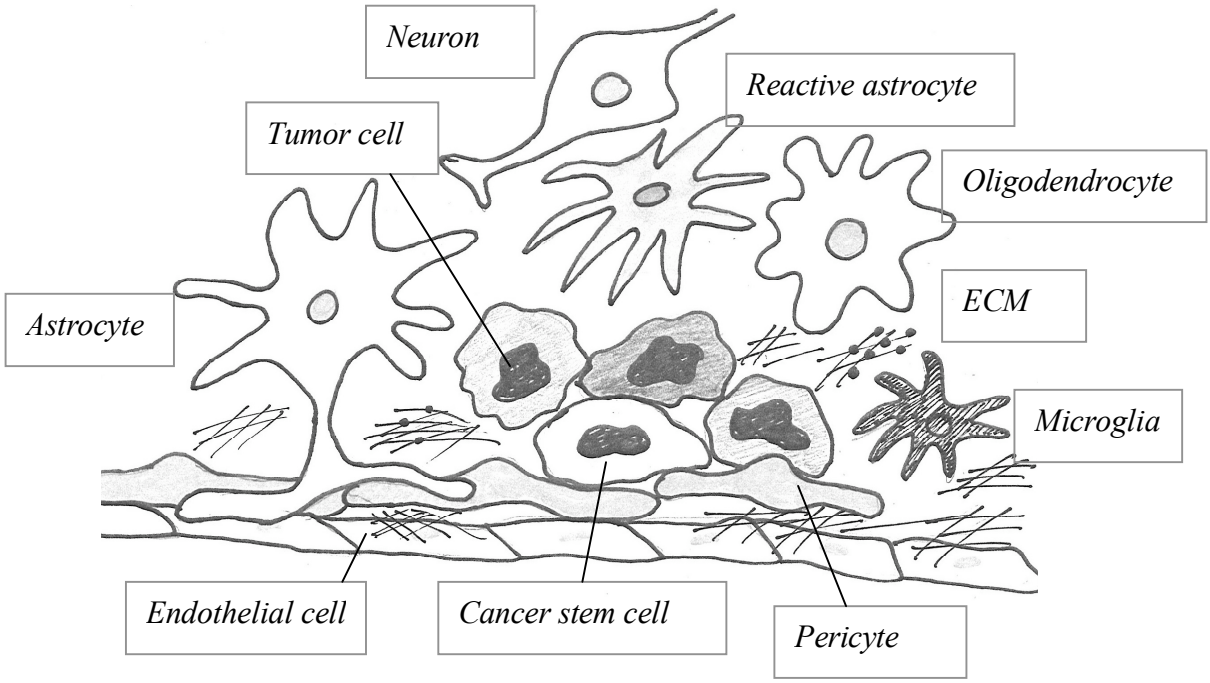

Figure 3. Brain tumor cells in a perivascular niche

2 AIMS OF THE STUDY

The aim of this work was to establish a transgenic mouse model harboring two of the most common aberrations found in human glioblastomas, lack of P53 function and overactive PDGF signaling.

This was done by generating genetically modified mice, over-expressing PDGFB in all cells of the astrocytic lineage including NSCs, and subsequently crossing them to *Trp53null* mice.

We aimed to use the transgenic mouse model to characterize the brain tumor phenotype in comparison to human glioblastoma, and to use it for elucidating the tumor cell of origin.

By analyzing this mouse model before brain tumor formation, we aimed to investigate the effects of excessive PDGFB and loss of p53, both individually and in combination, in order to dissect out what cellular changes precedes brain tumor development.

We also aimed to prove that genetic alterations common in human glioblastomas, here exemplified by PDGFB and p53, can influence and change the properties of both brain cells and brain vasculature.

3 RESULTS AND DISCUSSION

3.1 PAPER I: P53 SUPPRESSES THE SELF-RENEWAL OF ADULT NEURAL STEM CELLS

Many of the genes that have been found mutated in human cancers, also play important roles in controlling stem cell functions. Some of the characteristic and most important features of stem cells are their abilities to control self-renewal, quiescence, proliferation and differentiation in adult tissues. One of the proteins that are involved in all these functions is P53.

The P53 pathway is commonly inactivated in brain tumors. In glial tumors mutations or loss of *TP53* are found in tumors of all grades, indicating that loss of P53 is a tumor initiating event that occurs early in the tumorigenic process. Glial tumors often express neural stem cell markers, and several studies in animal models suggest that neural stem cells may be their cell of origin.

To investigate if the neural stem cells are affected by loss of P53, we used mice with a germ-line deletion of *Trp53*. Male *Trp53null* mice and *wt* mice at the age of 8-12 weeks were used in all experiments.

First we confirmed that the p53 protein normally is expressed in the lateral ventricular wall. Staining of wt mouse brain with an antibody against p53 revealed the presence of p53-expressing cells in the SVZ. Both Gfap+ and Musashi+ neural stem cells of the SVZ, as well as ependymal cells expressed p53. Less p53 expression was found in Doublecortin expressing neuroblasts and in the surrounding brain tissue.

Next, we compared the numbers of proliferating cells in the lateral ventricular wall of *Trp53-/-* and *wt* mice. This was done by *in vivo* BrdU labeling of adult mice, and subsequent analysis of the BrdU incorporating cells in brain sections. In the *Trp53-/-* SVZ, there was a significant increase in proliferating cells, not accompanied by a decrease in apoptosis. These results are in line with other studies where loss of p53 in the mouse SVZ also resulted in an increased proliferation rate, but no decrease in apoptosis (Gil-Perotin et al. 2006).

Then we went on to compare the *Trp53-/-* and *wt* neural stem cells *in vitro*, by preparing neurosphere cultures from the lateral ventricular wall. The neurosphere cultures were compared in their ability to form primary spheres and their ability to form new secondary spheres after dissociation. We found that the *Trp53-/-* neural stem cells displayed a significantly higher neurosphere-initiating capacity, and that the formed spheres grew faster. This implies an increased self-renewal capacity as well as an increased proliferation rate. There were also significantly fewer apoptotic cells in the *Trp53-/-* neurospheres, compared to *wt*. This decrease in apoptotic cells *in vitro* compared with the results from the TUNEL-stained tissue sections, may reflect differences in the amount of stress signals leading to a p53-induced apoptotic response in the cellular environments.

We also examined the multipotency of the neurospheres, and found that both *wt* and *Trp53-/-* spheres were able to form differentiated Gfap+ astrocytes, Tuj1+ neurons and O4+ oligodendrocytes. However, we did not measure the ratio of the different cell types and can therefore not exclude an altered differentiation pattern. Later reports have shown that neural stem cells lacking p53 form more Tuj1+ cells and are more prone to differente to a neuronal lineage (Armesilla-Diaz et al. 2009).

We also performed a gene expression analysis and compared the transcriptome of *wt* and *Trp53-/-* neurospheres. A microarray enriched for stem cell genes was used, and the analysis resulted in a list of more than 300 genes that were differentially expressed. Of these, the single most deregulated gene was *Cdkn1a*, which was down-regulated by 21-fold in *Trp53-/-* neurospheres compared to *wt*. The protein product, p21 is directly down-stream of p53 in the cell cycle regulatory pathway, and functions as a CDK inhibitor, preventing the cell from entering S-phase. Mice lacking p21 also display increased proliferation in the lateral ventricular wall (Kippin et al. 2005). One major difference between the *Trp53-/-* and *Cdkn1a-/-* mice is that after an initial increase in neural stem cell proliferation, the stem cells become depleted in the *Cdkn1a-/-* SVZ. This suggests that loss of p53 is necessary for maintaining the self-renewal capacity, and that this is separate from the cell-cycle regulating function. Results from mammary stem cells for example, show that p53 regulates stem cell polarity. Loss of p53 leads to an increase in symmetric, self-renewing cell divisions, thereby expanding the stem cell pool (Cicalese et al.2009).

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

Human gliomas often display excessive PDGF signaling, through over-expression and amplification of *PDGFRA* and through increased expression of the PDGF ligands. This results in both autocrine and paracrine stimulatory loops in the tumor tissue. LOH on chromosome 17p and mutations in *TP53* are often found together with over-expression of *PDGFRA* in both low-grade and high-grade gliomas, indicating that these aberrations are important early events in the tumorigenesis.

We aimed to establish a transgenic model for human glioblastomas, by over-expressing PDGFB in the *Trp53 null* mouse brain. We used a 1.8kb human *GFAP* promoter to direct the expression of the PDGFB transgene to neural stem cells as well as to cells of the astrocytic lineage. The transgenic construct contains a beta-galactosidase (betagal) reporter gene. By X-gal staining we found that the transgene was expressed in the embryonic telencephalon and in astrocytes of adult brain. Earlier work have shown that the 1.8kb *GFAP* promoter is active from E8.5 in mice (Andrae et al. 2001).

We then performed a more detailed analysis of the expression pattern by immunohistochemical staining for betagal, revealing a strong expression in the newborn mouse lateral ventricular wall. In the adult mouse brain, the expression of betagal was somewhat weaker. We found betagal-expressing cells in adult lateral ventricular wall, in the glia limitans and in scattered astrocytes throughout the brain. Immunohistochemical staining for Gfap on X-gal stained adult brain tissue, showed that the transgene expression co-localised with Gfap.

The *hGFAPpPDGFB* mouse strains were then crossed to *Trp53*+/- mice in several steps, to eventually obtain mice with the combined genotype PDGFB/p53-/-. These mice, together with their littermates of wt, PDGFB and p53-/- genotypes were followed for up to 18 months. The *Trp53null* mice developed lymphomas and sarcomas within the first half year of life, just as previously reported (Donehower et al. 1992). The PDGFB/p53-/- mice on the other hand, displayed a high incidence of brain tumors. In the two PDGFB/p53-/- mouse strains, 68% and 43% of the mice developed brain tumors at the age of 2-6 months, in addition to the *Trp53-/-* related lymphomas and

sarcomas. Mice over-expressing PDGFB in a *wt* background did not have any brain tumors.

The fact that over-expressed PDGFB needs to be combined with loss of p53 to induce brain tumors has not been shown before. Here, the tumors only formed in the adult mouse brain, although the transgene was highly expressed already in the embryo. In retroviral animal models, PDGFB induces gliomas when introduced both in embryonic and newborn mice (Appolloni et al 2009, Uhrbom et al.1998). This may reflect the targeting of different cell populations and the fact that retroviral insertions occur in the genome of those mice. Here, the tumor cell of origin is only capable of developing into tumors in the adult brain, and in a p53 dependant way.

The brain tumors that formed in our animal model, were of different sizes and were located in many areas of the brain. We found brain tumors engaging both the ventricular lining, SVZ, pial lining and subpial areas. Large macroscopic, and small microscopic tumors were found in both cerebrum, cerebellum and brain stem. This pattern of tumor location may imply that the tumor cell of origin also was located in the same areas. Several potential tumor initiating cells can be considered. Tumors may have originated from differentiated astrocytes that de-differentiated and formed tumors. Another explanation is that the NSCs from the SVZ, and perhaps also from cerebellar stem cell niches, gained migratory properties and formed brain tumors that spread in the brain tissue.

The brain tumors were analyzed by histology and by immunohistochemical staining for different cell lineage markers. Many tumors displayed features of human glioblastoma, pseudopalisading necrosis, microvascular proliferation and nuclear pleomorphism. Oligodendroglial histological appearance was very rare. The tumors were characterized by a robust expression of Pdgf receptor alpha on all tumor cells and Pdgf receptor beta on tumor vessels, indicating the presence of both autocrine and paracrine stimulatory mechanisms in the tumor tissue.

Comparison of the expression patterns of betagal in small and larger tumors in the ventricular area showed that betagal expression was present already in the smallest tumors, and that tumor cells in larger tumors expressed variable levels of the transgene.

A similar variability was seen in tumors stained for PDGFB. This may reflect that a subset of tumor cells are driving tumor growth by producing high levels of PDGFB.

We found that the tumors expressed many different cell lineage markers, exemplified by Gfap, Vimentin, Nestin, Map2, Tuj1, CNPase and F4/80. When comparing the small and large tumors we found that the neural stem cell marker Nestin was mainly expressed in the larger tumors. Expression of Gfap was seen in both small and large tumors. However, it is difficult to distinguish between Gfap+ tumor cells and Gfap+ infiltrating reactive astrocytes. We saw that many of the larger tumors partially lost Gfap expression, but retained betagal positivity. This was surprising, since the *GFAP* promoter drives expression of the transgene. The Gfap protein comes in several splice variants, and the tumors could produce a splice-variant of Gfap that is not detected by our antibody. In human brain for example, a GFAP-delta splice variant is specifically expressed in neural stem and progenitor cells (van den Berge et al. 2010).

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

We have generated a transgenic mouse model where over-expression of PDGFB in *Trp53-/-* mice (B+P53-/-) lead to glioblastoma-like brain tumors. Here, we used the same mouse model to study the brain, before tumor formation. To investigate how the two genetic changes contribute to brain tumor formation, we compared brains from B+P53-/- mice to brains from wt, B+ and p53-/- mice.

First, we wanted to find out if the neural stem cells were affected before brain tumor formation in the B+P53-/- brain. We know that loss of p53 increases the self-renewal capacity and proliferation of neural stem cells, but the effect of PDGF-B was not known. X-gal staining of neurospheres showed that the transgene was expressed in B+ and B+P53-/- NSCs. The proliferation rates of NSCs of different genotypes were compared by measuring of sphere size. We found that both p53-/- and B+p53-/- NSCs proliferated faster than wt NSCs. The neurospheres of all genotypes were multipotent, as they could all differentiate into astrocytes, oligodendrocytes and neurons.

To visualize Pdgfr-alpha expressing cells we crossed the transgenic mouse strain to a Pdgfr-alpha-GFP reporter mouse strain. Immunofluorescence staining for betagal of neurospheres generated from B+GFP and B+P53-/-GFP mice revealed co-expression of Pdgfr-alpha with the transgene, indicating a PDGF-B autocrine loop stimulating growth of the neural stem cells. By immunofluorescence stainings for different stem cell markers we also found the transgene to be co-expressed with Gfap, Olig2 and Sox2 in NSCs, the same markers that were found expressed in the brain tumors of B+P53-/- brain.

Next, we examined if neurospheres could be established from other regions of the brain than the SVZ. We have earlier shown that brain tumors develop in many different regions of B+p53-/- brains. One hypothesis is that the NSCs migrate out from the SVZ to form brain tumors in other locations of the brain. Cultures were prepared from lateral ventricular wall (SVZ), corpus callosum, hippocampus, frontal (cortical) and basal (brainstem) subpial region. Neurosphere cultures could be established from lateral

ventricular wall of all genotypes (wt, B+, P53-/- and B+P53-/-), and also from corpus callosum of B+P53-/- brains.

The B+P53-/- corpus callosum also contained an increased total number of cells, compared to wt. These were not oligodendrocyte precursor cells, since there was no increase in Olig2 expressing cells in this area. However, we found increased amounts of Pdgfr alpha expressing cells, and BrdU labeling experiments showed that the B+P53-/- corpus callosum contained proliferating Gfap expressing cells.

We went on to investigate if there was a change in the number of PDGF responsive cells also in other adult brain areas before tumor formation. We counted the number of Pdgf receptor alpha expressing cells and Pdgf receptor beta expressing vessels in SVZ, hippocampus, corpus callosum and subpial regions in frontal cortex and in basal brain at the brain stem level. Compared to wt, B+P53-/- brains had increased amounts of both Pdgfr-alpha expressing cells and Pdgfr-beta expressing vessels in SVZ and subpial regions in both cerebral cortex and basal brain.

The IHC stainings for Pdgfr-beta showed that there was a higher number of Pdgfr-beta expressing vessels in the B+P53-/- adult brains. Those vessels were also more prominent, with a wider lumen and thicker wall, and a strong Pdgf receptor beta expression. A similar phenotype was also seen in B+P53-/- retina, with pericyte-like but abnormal Pdgf receptor beta and ASMA expressing cells.

In summary, excessive PDGF signaling and loss of p53 leads to an abnormal expansion of the neural stem and progenitor cell pool as well as to a changed vasculature. The fact that tumors develop only in the adult brain and only on a p53-/- background with the accompanying vascular phenotype, indicates that the B+P53-/- dependant modification of the vascular niche is essential for glioblastoma development.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis I have shown that the combination of excessive PDGFB and loss of p53 induces high-grade gliomas in mice. Our transgenic model show that loss of p53 is necessary for the formation of glioblastomas by PDGFB in adult mouse brain.

Loss of p53 by itself leads to increased proliferation and increased self-renewal of neural stem cells. When adding excessive PDGFB expression to the *Trp53-/-* phenotype, the increased self-renewal and proliferation capacity are maintained. In addition, cells with NSC properties are found in a more widespread area of the brain.

These data suggest that the deregulated NSC niche can serve as the origin of glioblastomas. It is difficult to identify the cell of origin, since the transgene is expressed in both NSCs, glial precursor cells and in mature astrocytes of the brain. The NSCs of the SVZ in B+P53-/- mice may have migrated to other locations of the brain, to form tumors outside the SVZ. Alternatively, the combination of loss of p53 and excessive PDGFB could have induced dedifferentiation of mature astrocytes in the brain.

This hypothesis could be tested by crossing the *hGFAPpPDGFB* mice to a mouse strain with a conditional deletion of *Trp53*. By deleting *Trp53* only in the NSCs of adult SVZ, it would be very interesting to see if tumors were still forming in other areas of the brain than the lateral ventricular wall.

The importance of the pre-tumorigenic changes that we observed needs to be further addressed. Neural stem cells and cancer stem cells are known to depend on vascular niches, and perhaps one crucial effect of PDGFB together with loss of p53 is to change the perivascular niche, allowing for brain tumors to form. The question whether the tumor-initiating capacity of neural precursors/astrocytes depends on the surrounding tissue can be addressed by transplantation experiments. If we transplant tumor cells or neural stem cells from B+P53-/- mice, will they form similar tumors also in a wt brain?

The significance of this work is that by establishing this transgenic mouse model of human glioblastoma, we have shown that the combination of loss of p53 and over-

expression of PDGFB is sufficient for brain tumor formation. Secondly, by comparing the phenotypic changes in brains of different genotypes with the formed tumors we will be able to figure out what mechanisms are the key events, and thereby the potential targets for therapy.

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