CHAPTER 1

General introduction and thesis outline
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GENERAL INTRODUCTION

Glioblastoma

Malignant gliomas constitute a spectrum of clinicopathological entities, from low-to high-grade malignancies, and almost all low-grade tumors eventually progress to high-grade malignancy.\(^1\) Traditional pathological approaches divide adult gliomas into astrocytomas, oligodendrogliomas and mixed oligoastrocytomas that is largely based on the predominant glial cell type (astrocytes or oligodendrocytes) present in tumors and the protein expression pattern as seen in immunohistochemistry (IHC).\(^1\) Furthermore, different grades with increasing malignancy are distinguished, World Health Organization (WHO) grade II, grade III (anaplastic) and grade IV (glioblastoma, GBM).

GBM is the most aggressive brain tumor characterized by diffuse infiltration of the brain parenchyma, aberrant proliferation, resistance to chemotherapy and radiation, and recurrence after surgical resection.\(^1\), \(^2\) In a clinical setting WHO grading is an important component among a combination of criteria used to predict response to therapy and outcome.\(^2\) Patients with WHO grade II tumors typically survive for more than 5 years and those with grade III tumors survive 2-3 years.\(^2\) GBM has the worst prognosis in the range of 12-15 months, despite multimodality treatment consisting of open craniotomy with surgical resection of as much of the tumor as possible, followed by concurrent and sequential chemo/radiotherapy.\(^3\)

The identification of a small fraction of cells with neural stem cell-like characteristics in GBM called glioma stem cells (GSCs), has opened up new avenues and strategies for better treatment.\(^4\), \(^5\) These GSCs express genes that are associated with multipotent neural stem cells (NSCs), possess multi-lineage differentiation potential, and are considered key drivers of tumor proliferation.\(^5\), \(^6\) Moreover, GSCs were shown to be resistant to radiation that may be related to more efficient DNA repair in GSCs compared to non-stem cancer cells.\(^7\) Interestingly, in xenograft animal models these GSCs faithfully reproduce parental GBM tumor pathology unlike traditional GBM cell lines cultured in serum. The GSC compartment is intensively investigated in order to identify pathways that control the self-renewal potential in GBM providing new targets for hopefully more successful treatment.

Molecular subtypes in GBM

Over the past two decades, extensive cytogenetic and molecular genetic studies have identified a number of recurrent genetic alterations and chromosomal abnormalities in malignant gliomas, particularly in GBM. Advances made in molecular technologies such as high-density microarray and genome sequencing, have made it possible to evaluate the genetic and epigenetic changes in these tumors at the genome-wide level.\(^8\), \(^9\) Recent
gene expression profiling studies have revealed molecular subtypes of high grade gliomas (grade 3 and 4) by Phillips et al\textsuperscript{10}, and of GBM by Verhaak et al\textsuperscript{11}, based on the preferential expression of genes characteristics of neural progenitor cells (proneural, PN), neurons (neural, N), proliferative cells and receptor tyrosine kinase activation (classical, CLAS), or mesenchymal tissues (mesenchymal, MES). Comparing the classification schemes of the two groups revealed particularly strong agreement in the gene signatures associated with the PN and MES subtypes.\textsuperscript{12}

Although still under evaluation, the different subtypes have been reported to have prognostic value. GBMs in the MES sub-class are aggressive, invariably coincide with disease recurrence, and are resistant to treatment modalities, whereas patients with the PN signature perform better in the clinic.\textsuperscript{10, 11} This indicates that the aggressiveness of GBM may be governed by genes that are involved in cell fate choice during neurogenesis (PN) or alternatively gene expression patterns that resemble mesenchymal cells (MES). Interestingly in some patients with recurrent disease a shift from a PN tumor into a MES subtype was observed and is assumed to be induced by therapy.\textsuperscript{10} Hence, genes that control subtype specificity could provide important novel targets for therapy.

A number of genes have thus far been linked with the different subtypes. For example, mutations in isocitrate dehydrogenase (IDH1), neurofibromatosis type 1 (NF1) and enhanced EGFR expression predominantly correlated with PN, MES and CLAS subtypes, respectively.\textsuperscript{11} Other histological markers reported to associate with a specific subtype include OLIG2, DLL3 for PN, and YKL40, CD44 for MES.\textsuperscript{10} Of note, other common genetic defects in GBM could not be assigned to a specific subtype, such as alterations in p53 and PTEN, and are seen in multiple subtypes.

**The mesenchymal GBM subtype and mesenchymal transition**

As mentioned, the mesenchymal GBM subtype is associated with the highest degree of invasiveness and aggressiveness and was linked with the worst prognosis. A number of transcription factors, C/EBP-\(\beta\) and STAT3 and more recently transcriptional coactivator TAZ, have been identified as master regulators of the mesenchymal phenotype in GBM.\textsuperscript{13, 14} However, in addition to these transcription factors it is conceivable that also autocrine and paracrine interactions involving the microenvironment of tumor cells will have a large impact on subtype status and tumor aggressiveness, similar as has been found for epithelial tumors.

Changes in cell phenotype between the epithelial and mesenchymal states, defined as epithelial-mesenchymal (EMT) and mesenchymal-epithelial (MET) transitions, have been recognized to play a key role in embryonic development, and more recently in the pathogenesis
of cancer and other human diseases.\textsuperscript{15, 16} EMT is a complex molecular and cellular program by which epithelial cells shed their differentiated characteristics, including cell-cell adhesion, planar and apical-basal polarity, and acquire instead mesenchymal features, including motility, invasiveness and a heightened resistance to apoptosis.\textsuperscript{16} The EMT process can be monitored by determining a loss of epithelial cell markers such as E-cadherin and gain of mesenchymal markers such as Vimentin and Fibronectin. The role of EMT in cancers was initially evidenced by the observation that colorectal tumor cells at the invasive front display signs of WNT pathway activation associated with loss of E-cadherin.\textsuperscript{17} Subsequently, the Weinberg lab has provided a strong basis for EMT as an important mechanism driving tumor cell invasion and metastatic disease in breast cancer.\textsuperscript{18} Both EMT and MET appear to have crucial roles in the tumorigenic process. In particular, EMT was found to contribute to invasion, metastatic dissemination and acquisition of therapeutic resistance. MET-the reversal of EMT- seems important during the colonization step of tumor cells at secondary tumor sites.\textsuperscript{16, 19, 20} In many cases EMT-inducing signals originate from stromal cells in the tumor, such as epidermal growth factor (EGF) and transforming growth factor-β (TGF-β), which on their turn activate a number of EMT-inducing transcription factors, such as SNAIL1, SNAIL2/SLUG, ZEB1, TWIST.\textsuperscript{18} Upon activation these transcription factors orchestrate the EMT process. Moreover also a link between mesenchymal transdifferentiation and stemness in mammary epithelial cells has been found, providing a conceptual integration of EMT with the cancer stem cell (CSC) model.\textsuperscript{21}

In GBM the process of mesenchymal transdifferentiation has been hardly investigated. A small number of studies have examined EMT transcription factors in GBM. TWIST expression has been found in GBM and was reported to promote invasion.\textsuperscript{22} The involvement of TWIST in activating mesenchymal transition in GBM cells was demonstrated and associated with enhanced expression of SNAIL2, MMP2, HGF, FAP and FN1.\textsuperscript{23} Furthermore siRNA-dependent silencing of SNAIL1 was found to inhibit the invasive properties of U87 cells.\textsuperscript{24}

A possible connection between mesenchymal transition and the MES subtype in GBM has remained largely elusive thus far. Recently, Cooper and coworkers reported that the MES subtype correlated to GBMs with high necrosis, necrosis being a known biological and prognostic significant feature of GBM.\textsuperscript{25} The authors show further a strong link between high necrosis and the expression levels of C/EBP-β and STAT3 in GBM tumor cells using IHC suggesting a possible link between necrosis and activation of the MES subtype transcription factors. However, the authors did not identify possible extracellular stimuli involved in mesenchymal differentiation of GBM. A first indication of such a relationship in literature may be provided by the recent finding of canonical WNT/β-catenin signaling being able to induce EMT in GBM cells involving the activation of ZEB1.\textsuperscript{26}
Triggers for EMT: TGF-β and hypoxia

In carcinomas both TGF-β and hypoxia have been discovered as important inducers of EMT. TGF-β represents a member of a large family of cytokines that include the bone morphogenetic proteins (BMPs), nodals and activins, which are involved in the regulation of embryonic development and tissue homeostasis. TGF-β signalling is highly active in high grade gliomas and elevated TGF-β activity has been associated with poor clinical outcome in this deadly disease. TGF-β regulates these different responses via binding to specific membrane receptors (TGF-β-RІ/ІІ/ІІІ) with serine/threonine kinase activity subsequently leading to the phosphorylation of SMAD2 and SMAD3. The phosphorylated SMADs together with SMAD4 are translocated into the nucleus resulting in transcriptional activation of many target genes.

Vascular hyperproliferation and necrosis are hallmarks of GBM and areas of necrosis are thought to represent regions of hypoxia. The hypoxic microenvironment stimulates adaptive transcriptional responses primarily involving the hypoxia-inducible transcription factors (HIFs), which regulate pathways including glycolysis, tissue invasion and metastasis, angiogenesis, genetic instability, immortalization, growth-factor signalling, apoptosis and pH regulation. Hypoxia-induced EMT has been linked to the HIF-signalling pathway mainly in epithelial cancers. The molecular network underlying this phenomenon is complex and not fully understood, although the activation of HIF-1 and 2 appear essential resulting in the up-regulation of EMT-associated transcription factors or repressors such as TWIST, SNAIL, SLUG and ZEB. Moreover hypoxia and HIFs have been found to activate pathways known to regulate EMT such as TGF-β, Notch, NF-κB, Wnt/β-catenin, and Hedgehog. As mentioned earlier, in GBM correlation between necrosis and angiogenesis have been associated with the MES subtype and the expression of related genes including C/EBP-β, C/EBP-δ and STAT3.

Glioblastoma stem cells (GSCs), differentiation and invasion

GSCs were shown to possess enhanced tumor initiation potential in comparison to non-GSCs. In vitro culture of GSCs in serum free medium containing bFGF and EGF produces spherical floating structures, named neurospheres. GSCs can differentiate into cell types resembling neurons, astrocytes and oligodendrocytes similar to the tri-lineage potential of normal NSCs. In comparison to differentiated counterparts, GSCs have been shown to be highly resistant for chemo- and radiotherapy, indicating that these cells may be responsible for tumor relapse after therapy. Multiple markers, such as CD133, CD15, CD44 and several others, have been proposed of being selectively expressed in GSCs, allowing antibody-based isolation techniques to enrich for GSCs. However, unlike hematopoietic stem cells and leukemic stem cells which can be sorted almost as pure populations based
on the expression of well characterized cell surface markers, the sorting of bona fide GSCs or NSCs population in pure forms still remains a technical challenge.

One of the major reasons for the poor prognosis associated with GBM is its highly invasive growth pattern in to the normal brain parenchyma, which limits the efficacy of surgical intervention, despite that surgical debulking remains the mainstay treatment strategy in GBM.\textsuperscript{43, 44} Several studies have indicated enhanced invasive potential of GSCs and their involvement in relapse of GBM.\textsuperscript{45, 46} However, the role of mesenchymal transition and differentiation in tumor cell migration and invasion in GBM has remained largely unexplored.

\textbf{AIM AND OUTLINE OF THE THESIS}

Targetting the molecular mediators of migration and invasion in GBM might be of potential benefit in improving the poor prognosis associated with this deadly disease. The aim of the research described in this thesis was to investigate if mesenchymal transition could be induced in GBM by external stimuli and affect the invasive behavior of GBM, and to understand the underlying molecular mechanisms involved in this process. In addition, in more general terms the role of the differentiation state in determining the invasive capacity of GBM cells was examined.

For our studies we generated a large panel of cell lines from fresh GBM surgical material. \textbf{In Chapter 2} we describe the generation and characterization of 6 newly generated GBM cell lines propagated as neurospheres from a panel of 33 cell lines. These cell lines were tested for stem cell and differentiation markers and were further divided in to the three main molecular subclasses, CLAS, PN and MES, based on expression levels of proteins previously found to be associated with these subclasses. The subtype status was compared between cells in vitro, upon intracranial implantation and with the corresponding patient material, in order to evaluate subtype stability under these different conditions.

\textbf{In Chapter 3} we review the effects of TGF-β specifically on the formation and progression of brain tumors, in particular gliomas and GBM. We briefly describe the role played by TGF-β in mediating some key events associated with gliomagenesis and glioma progression such as angiogenesis, vasculature, invasion, drug/radio resistance and stemness. Therapeutic approaches that have been explored in preclinical models are described, and clinical studies with TGF-β signaling-targeting agents, and future perspectives are discussed.

\textbf{In Chapter 4} we investigated the possible role of TGF-β in inducing a mesenchymal phenotype in GBM. The effect of TGF-β was tested on mesenchymal differentiation, migration/invasion capacity and tumor forming ability using in vitro established and newly generated GBM cell
lines as well as in orthotopic mouse models. The chemical inhibition of TGF-β signaling and siRNA-dependent silencing of downstream components were employed to determine the mechanistic link between TGF-β and induction of mesenchymal phenotype. Finally, we also examined the connection between TGF-β pathway activation and mesenchymal marker expression in GBM patient material.

In Chapter 5, we studied the possible role of hypoxia in inducing a mesenchymal shift and enhancing migratory potential in GBM cells. The hypoxia-induced effect could be inhibited by the chemical inhibitor digoxin and by ShRNA and SiRNA mediated silencing of particular key components of the HIF pathway. Further we provide evidence for the occurrence of a hypoxia-mediated mesenchymal shift in patient material. Taken together in chapter 4 and 5 we discovered the involvement of microenvironmental factors represented by TGF-β and hypoxia in regulating a mesenchymal shift, which can result in locally enhanced mesenchymal properties in GBM, thereby enhancing the invasive phenotype of these cells.

In Chapter 6 we made an attempt to assess whether routine immunohistochemistry (and FISH) on formalin-fixed paraffin embedded (FFPE) GBM tissue samples could be utilized for GBM subclassification. We were able to sub-classify two-third of the 123 archival primary and adult GBM cases into predefined molecular subclasses through the assessment of protein expression patterns. The protein signatures-based subclassification was found to be in agreement with an established PCR-based metagene analyses.

In Chapter 7 we examined the relationship between differentiation state and invasive properties of GBM cells. Therefore, we compared the invasive and neurosphere forming potential of GBM cells cultured either on serum-free or serum-differentiated conditions in in vitro and in vivo experiments. Also the role of Matrix metalloproteinase-9 (MMP9) in tumor invasion was examined. Further in this study we also tested if the differentiated cells can revert back to a stem cell/ undifferentiated state as it is known that a stem cell state is crucial in maintaining the proliferative potential and sustaining tumor growth.

In Chapter 8 we tested the credibility of CD133 (Prominin1) in identifying and sorting GSCs from several GBM cell lines. We extended our study in testing three characteristics predominantly associated with GSCs namely- neurosphere formation, multilineage differentiation and DNA damage repair potential. The effect of differentiation on CD133 levels as well as radiation induced DNA damage and apoptosis was also tested in this study.

In Chapter 9 the findings of this thesis are summarized, followed by a general discussion and future perspectives of molecular mediators regulating mesenchymal transition and invasion in GBM and its potential in therapy and diagnostics.
REFERENCES


