Glioma as an Ecosystem

Studies of Invasion, Onco-miR Addiction and Mast Cell Infiltration

JELENA PÔLAJEVA
Despite recent advances in oncology and extensive research efforts, gliomas remain essentially incurable. Glioblastoma multiforme (GBM, WHO grade IV) is the most common glioma and may arise de novo or progress from a lower-grade lesion. GBM is characterized by invasive growth, aberrant angiogenesis and necrosis. The heterogeneity of GBM is further complicated by the contribution of the inflammation that is facilitated by immune cells that reside in and infiltrate this immuno-privileged organ.

One of the cell types present in the tumor microenvironment are mast cells (MC) that accumulate in the tumor in a grade-dependent manner. GBM cells secrete a plethora of cytokines acting as chemoattractants in MC recruitment and to a lesser degree induce MC proliferation in situ. Expression of one of the cytokines secreted by GBM cells - macrophage migration inhibitory factor (MIF) - correlates with MC accumulation in vivo.

GBM cells invade the surrounding parenchyma making complete resection impossible. Here, migration was studied with the focus on RAP1 and its negative regulator RAP1GAP. Activation of RAP1 signaling by lentiviral silencing of RAP1GAP lead to decrease in cell migration and a shift in expression of SOX2 and GFAP, presumably enhancing stem cell phenotype.

MicroRNAs are small non-coding RNAs known to regulate the mRNA network. miR-21 is highly overexpressed in the majority of cancers including GBM. Its expression is strictly regulated during embryonic development of the brain. SOX2 is co-regulated with miR-21 demarcating a cell population with neural/glial progenitor/stem cell properties. In an experimental mouse model, expression of miR-21 can be sustained by forced expression of PDGF-BB leading to gliomagenesis. GBM cells seem to be addicted to oncogenic properties of miR-21 as its knockdown leads to extensive apoptosis. This observation combined with the fact that miR-21 is absent in the normal adult mammalian brain suggest miR-21 to be an excellent therapeutic target.

Effects of conventional therapy (surgery combined with radiochemotherapy) on prolonging patient survival have reached a plateau. New effective personalized therapeutic modalities need to be designed and implemented. Targeting the tumor microenvironment as well as cell intrinsic properties like invasive potential, stemness and onco-miR addiction studied in this thesis will hopefully lead to efficient disruption of GBM’s aberrant ecosystem.

**Keywords:** glioblastoma, mast cell, miR-21, RAP1, CXCL12, SOX2
“If the brain were so simple we could understand it, we would be so simple we couldn’t.”
Lyall Watson (1939-2008)
List of Papers

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


* These authors contributed equally to this work

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<td>BBB</td>
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<td>CIMP</td>
<td>CpG island methylator phenotype</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>FISH</td>
<td>fluorescence <em>in situ</em> hybridization</td>
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<td>GAP</td>
<td>GTPase activating protein</td>
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<td>GBM</td>
<td>glioblastoma multiforme</td>
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<td>GEF</td>
<td>guanine exchange factor</td>
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<td>GSC</td>
<td>glioma stem cell</td>
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<td>HIF-1α</td>
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<td>HPV</td>
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<td>IDH</td>
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<td>MC</td>
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<td>neural stem cell</td>
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<td>PDGF</td>
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<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
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<td>RTK</td>
<td>receptor tyrosine kinase</td>
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<td>SCF</td>
<td>stem cell factor</td>
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<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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<td>siRNA</td>
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<td>TGF-β</td>
<td>transforming growth factor β</td>
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<td>TMA</td>
<td>tissue microarray</td>
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<td>TME</td>
<td>tumor microenvironment</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>WHO</td>
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Glioma

Clinical overview

The World Health Organization (WHO) categorizes gliomas into astrocytomas, ependymomas and oligodendrogliomas, but this does not necessarily mirror the cellular origin of these neuroepithelial tumors. Despite advances in multimodal molecular techniques that hold great promise in establishing personalized treatment schemes and improving prognostic accuracy, diagnosis of gliomas is still based on the microscopic evaluation of hematoxylin and eosin stained slides (Orr and Eberhart 2012). The histopathological grading system takes into account morphological features of the specimen and subdivides astrocytomas into four grades, grade I being least malignant. Histopathological hallmarks of glioblastoma multiforme (GBM), grade IV, are vascular proliferation, diffuse infiltration and necrosis (Figure 1.). Necrosis is caused by hypoxia and induces inflammation which in turn enhances cell migration beyond the borders of resectable tumor, at times as far as into contralateral hemisphere of the brain. In the tumor margin, infiltrative tumor cells are intermixed with normal parenchyma impeding curative surgery. Disruption of the so called “blood brain barrier” (BBB) is caused by faulty angiogenesis and leads to local edema (Adamson et al 2009, Bonavia et al 2011, Lathia et al 2011).

The majority of GBMs occur de novo and are termed primary. They are clinically indistinguishable from secondary GBMs that arise from a lower grade lesion, but have a later age of onset and a distinguishably different profile of genetic alterations (Louis et al 2007, Schetter et al 2008).

The cause of GBM remains unknown. However, some environmental risk factors like exposure to carcinogens have been identified. Further, inherited mutations in tumor suppressor genes as in neurofibromatosis syndrome predispose for GBM. The patients commonly present to the clinic with headaches, seizures and progressive focal loss of neurologic activity (Adamson et al 2009).

Despite advances in surgical techniques and radiotherapeutical approaches, the median survival is about 15 months and the two-year survival rate barely exceeds 26%. The incidence of GMB is in the range of 7 individuals per 100 000 adults, affecting mainly the elderly and men more commonly than women (with a ratio of 3:1) (Stupp et al 2005).
Figure 1. Schematic overview of heterogeneity within GBM with distinct areas like invasive edge, perivascular niche and hypoxic/necrotic region. GBM is comprised of different cell types: cells that form the bulk of the tumor, glioma stem cells (GSC), mast cell (MC), resident and infiltrating immune cells and angiogenic vascular cells. Based on Paper I and (Charles et al 2011, Hanahan and Coussens 2012, Lathia et al 2011).

Cellular aspects

A number of theories teach us that diversity within a given system provides an evolutionary advantage to its participants (Bonavia et al 2011). The simplistic view that tumors are composed of a homogenous malignant cell mass has been replaced by elaborate theories depicting a tumor as an organ with complex interactions between different compartments as well as hierarchical relationships between tumor cells (Hanahan and Weinberg 2011). Gliomas reside in an immune-privileged milieu that facilitates their key hallmark features such as vascularization, invasion, unauthorized proliferation and chemoradiotherapy resistance (Figure 1).

Glioma stem cells

Thus far, gliomas have been clinically classified based on their histological features, not necessarily the cell of origin. After being originally discovered about a decade ago (Hematti et al 2003, Ignatova et al 2002, Singh et al 2004), glioma stem cells (GSC) are characterized as cells that display a number of stem cell characteristics such as self-replication, capacity to dif-
ferentiate into multiple lineages and potential to generate tumors upon inoculation in immunodeficient mice. In their natural habitat they are responsible for sustained growth of the bulk of the tumor. Therefore, even if glioma cells were genetically homogeneous (which is not the case), the tumor would remain phenotypically heterogeneous due to presence of cells in different differentiation stages. This hierarchical model provides rationale as to why accurate diagnosis and grading of this kind of tumor is complicated (Vescovi et al 2006).

GSCs rely on the surrounding niche to provide structural support and cues for maintenance of their undifferentiated phenotype. Under hypoxic conditions (in the hypoxic niche) increased expression of hypoxia-inducible factor 1α (HIF-1α) precedes enhanced self-renewal of GSCs and contributes to the retention of their undifferentiated state (Soeda et al 2009). In the perivascular niche, endothelial cells have been shown to participate in nitric oxide (NO) production that, via Notch signaling, enhances platelet-derived growth factor induced GSC tumorigenic characteristics (Charles et al 2010). In other brain tumors such as medulloblastoma and ependymoma, endothelial cells were required for self-replication and propagation of brain tumor stem cells as depletion of blood vessels led to loss of the self-renewing cells (Calabrese et al 2007).

In turn, GSCs produce cytokines that act in both an autocrine and paracrine fashion to enable invasion, immune suppression and angiogenesis. For example, autocrine interleukin 6 (IL-6) signaling promotes GSC survival whereas IL-6 inhibition has been shown to lead to prolonged survival in tumor-bearing mice. The data obtained from experimental mouse models were in line with observations in humans, where increased IL-6 and receptor levels correlated with poor patient survival (Wang et al 2009). Interleukin 10 (IL-10) and transforming growth factor β (TGF-β) secreted by GSCs in vitro could potentially account for the immunosuppressive properties of gliomas (Qiu et al 2011). Intriguingly, GSCs have been demonstrated to be capable of transdifferentiation into endothelial cells and formation of vessels (Ricci-Vitiani et al 2010).

Current therapies targeting the tumor mass fail to eliminate GSCs due to their slow cell cycle and expression of drug export pumps such as ABCG2 (Bleau et al 2009, Stiles and Rowitch 2008). Until efficient GSC-specific treatment modalities have been designed and proven effective, the presence of GSCs in the glioma ecosystem remains a life-threatening feature, as these cells are resistant to therapy and are also capable of regenerating the tumor post surgery (Hanahan and Weinberg 2011).

Glioma vasculature

Induction of angiogenesis, i.e. sprouting of new vessels from pre-existing ones, is a hallmark of cancer progression (Hanahan and Weinberg 2011), and
gliomas are no exception to this, as they are highly vascularized. In fact, microvascular proliferation and endothelial hyperplasia are particularly evident in the histopathology of high-grade gliomas (Wen and Kesari 2008). This is caused by continuous pro-angiogenic stimuli originating from glioma cells.

A unique feature of the brain is its highly restricted blood circulation facilitated by intimate interplay of endothelial cells, pericytes and astrocytes (Armulik et al 2010, Kim et al 2006a). However, the BBB is locally disrupted in gliomas due to a number of factors. Induced by hypoxia, oncogene signaling or released from extracellular matrix (ECM) and subsequently activated by proteases (e.g. matrix metalloproteinase 9 (MMP-9)) (Hanahan and Weinberg 2011), vascular endothelial growth factor (VEGF) causes stimulation of endothelial cell migration and proliferation as well as increasing vascular permeability leading to vessel dysfunction, leakiness and edema (Jain et al 2007).

In cancer, besides their primary function of oxygen and nutrient supply, vascular endothelial cells produce paracrine trophic factors and facilitate the infiltration of immune cells. Pericytes endow the endothelial cells with structural support (Hanahan and Coussens 2012).

Other cellular components of the tumor microenvironment

Although the immune-privileged status of the brain has been largely accepted, it does harbor certain immune cell types. Evidence of the contribution of these immune cells in assisting the host in tumor suppression at an early stage and immunosuppressive response at late tumor stages is beginning to emerge. Microglia, resident macrophages in the brain, account for the largest inflammatory cell group in gliomas. Conditioned by glioma-released factors, microglia immune functionality reduces. Instead, intercellular interaction between glioblastoma cells and microglia results in enhanced tumor growth and invasion (Charles et al 2011).

In addition to microglia (and cell types described above), TME contains, but is not limited to, leukocytes, including mast cells (MC) (Paper I). The observation that leukocyte infiltration increases with grade makes it evident that immune cells are recruited to the tumor site to assist in tumor growth and invasion (Farmer et al 1989). However, the contribution of the immune system at earlier stages is less clear.

The well-known immunosuppressive phenotype of gliomas is partially executed by regulatory T cells. This CD4-positive subset of cells aids glioma in restriction of tumor-infiltrating lymphocyte function by inhibiting their proliferation and pro-inflammatory cytokine production (Fecci et al 2006).
Mast cells

MC origin and recruitment

Mast cells (MC) were initially described at the end of the 19th century and characterized by the presence of granules that reacted with aniline dyes (Ehrlich 1878). These granules make up a large fraction of cell’s weight and endow an MC with its key function – release of mediators upon stimulation. (Gurish and Austen 2001). MCs originate from hematopoietic stem cells, leave the bone marrow as committed precursors to circulate and home to target tissues where they receive proliferative and differentiating cues and finally mature into granulated cells (Ribatti and Crivellato 2012). MCs reside in the surfaces that serve as entry points for infection such as skin, mucosa of the gastrointestinal, respiratory, and genitourinary tracks, surrounding lymph nodes, nerve bundles and blood vessels. Resident MCs are also present in the brain under physiological conditions where they reside in the epithalamus, more specifically the median habenula (Silverman et al 2000). Just 13 years after their original discovery, a student of Paul Ehrlich, Otto Westphal illustrating (using the same kind of metachromatic staining) the presence of MCs in the periphery of a developing tumor (Westphal 1891).

Being produced by a variety of cells, e.g. neurons (Leon et al 1994), endothelial and stromal cells, stem cell factor (SCF) plays a crucial role in the maturation and chemotactic process of MCs. In a hepatocarcinoma model, MC infiltration was abolished in SCF-knockdown tumors. Usage of antibodies against KIT (SCF receptor) in SCF wt tumors gave similar results, pinpointing a specific role for this axis in MC recruitment (Huang et al 2008). Other cytokines that play a role in MC recruitment specifically to the brain tumor microenvironment include nerve growth factor, TGF-β1 (Silver et al 1996), and as of recently CXCL12 (Paper I) and macrophage migration inhibitory factor (MIF) (Paper II).

MC activation

Massive (IgE-dependent) degranulation can occur via stimulation of FCεRI receptor in response to allergens, defense against certain parasites but also in other inflammatory conditions like asthma. More selective and limited degranulation has also been described and may occur under chronic inflamma-
tion conditions, including rheumatoid arthritis, multiple sclerosis and cancer. Alternative pathways include activation of, for example, KIT, TLR or FcγRIII receptors (Ribatti and Crivellato 2012). MCs can also be activated by basic compounds like substance P, compound 48/80 and pharmacological substances, e.g. morphine (Metcalf et al 1997). Secretory granules contain a variable composition of histamine, proteases, cytokines and growth factors implanted into a proteoglycan meshwork matrix. In general, histamine and major MC proteases, like tryptase and chymase are stored in the granules in a preformed state, whereas growth factors and more than thirty different cytokines are synthesized de novo upon stimulation (Ribatti and Crivellato 2012).

A different extent of degranulation and versatile mediator composition endows MCs with an ability to tune their responses based on the stimulus.

**MC role in tumor growth**

Like in other pathologies, MC involvement in carcinogenesis is thought to be due to their ability to primarily secrete mitogenic and proangiogenic factors and remodel ECM but also by modulating immune response.

Mouse models have been instrumental in elucidating the specific contribution of mast cells to either tumor initiation or progression. For example, pro-inflammatory MCs (as well as their precursors) are present in adenomatous polyps, preneoplastic lesions of colon cancer. After pharmacological intervention in wt mice and in chimeric animals with MC development defects a marked remission of existing polyps was observed (Gounaris et al 2007).

In a Myc-driven pancreatic β-cell tumor model secretion of the chemokine CCL5 led to a rapid MC recruitment to the tumor. This was shown to be essential for tumor expansion as degranulation inhibition (by chromolyn) resulted in rapid hypoxia and apoptosis of the tumor and endothelial cells (Soucek et al 2007).

Another elegant example of MC involvement at the early stages of tumor development is the study by Coussens and coworkers on MC accumulation in hyperplastic and dysplastic lesions of squamous cell carcinoma. In this HPV (human papillomavirus) 16 infected transgenic mouse model MC presence and release of tryptase and chymase in the invasive edge also coincided with angiogenic switch and MMP-9 activation. Notably, in MC-deficient HPV16 mice, neoplastic angiogenesis was abolished demonstrating MC participation in tumor angiogenesis (Coussens et al 1999).

As described above, MCs can contribute to tumor progression by directly affecting the growth of tumor cells. Indirect effects on tumor phenotype via enhanced angiogenesis, ECM remodeling and immune cell recruitment can, however, be just as critical.
MC contribution to angiogenesis and tumor invasion

There have been numerous examples of associations between MC numbers and neovascularization in human tumors, namely, in uterine cervix cancer, laryngeal carcinoma, melanoma and various cancers of gastrointestinal tract (Ribatti and Crivellato 2012).

MCs are implicated in angiogenesis due to secretion of potent vascular mediators such as VEGF, angiopoietin-1, IL-8, histamine and heparin. The former can also be released from ECM by MC-secreted proteases (Hanahan and Coussens 2012).

Effects of MC mediators on ECM remodeling and increased invasive potential of a tumor go hand in hand. MCs are reservoirs of ECM degrading agents, *i.e.*, chymase, tryptase and metalloproteases. As demonstrated by Coussens *et al* (Coussens *et al* 1999), there is a link between proangiogenic and remodeling effects of MCs in an experimental setting, emphasizing the variety of tasks MC can take upon themselves in a developing TME.

MC participation in immune modulation

MCs possess the ability to orchestrate responses of both innate and adaptive arms of the immune system. In the cancer setting, their role in modulating immune response may be pro- or antitumorigenic. Once again, the outcome depends on the composition of the MC granules. Although the immunosuppressive activity of MCs is poorly understood, they can exert this function by secreting inhibitory cytokines like IL-10 or by assisting regulatory T cells (Tregs) in contributing to immune tolerance (Lu *et al* 2006).

Counteractive functions of MCs in dysplasia

Clinical association studies have correlated MC infiltration with poor prognosis in, for instance, melanoma, Hodgkin’s lymphoma, oral squamous carcinoma and prostate cancer. Interestingly, the association of MC infiltration with good prognosis has also been observed. Tumors where increased MC numbers correlate with a good prognosis include non-small-cell lung carcinoma, breast and ovarian cancers (Ribatti and Crivellato 2009). Due to the versatility of MC mediators, the net effect of MC presence will depend on the responsiveness of tumor cells and other components of TME to secreted molecules.

Data regarding the infiltration and, in particular, the function of MCs in tumors of the brain, remain rudimentary. The presence of MCs in meningiomas was demonstrated in 1968, with high mast cell numbers particularly in the secretory meningoma subtype (Tirakotai *et al* 2006). We were the first to demonstrate grade-dependent accumulation of MCs in glioma (Paper I and II).
Molecular chaos of glioma

Molecular subtypes of glioblastoma

Cellular processes are tightly regulated in terms of cell survival, proliferation and migration. Two important hallmarks of cancer cells are that they are able to evade control mechanisms and are self-sufficient in growth signals (Hanahan and Weinberg 2000). Historically gliomas have been classified based on their histopathology but recently in order to address intertumoral heterogeneity more efficiently, studies attempting to divide high-grade gliomas into molecular subclasses have been conducted. A pioneering study was performed by Heidi Phillips and colleagues, where gene expression profiles of newly diagnosed cases were determined using microarray technology. The grouping of samples based on aberrations in signaling pathways resulted in tumor signatures with obvious prognostic value (Phillips et al 2006). For example, the proneural subtype prevailing in younger patients, is characterized by intact PTEN (phosphatase and tensin homolog) locus and is associated with longer survival when compared to proliferative and mesenchymal subtypes. The hallmarks of the latter include loss of PTEN and activation of Akt. Lately, our understanding of distinct molecular signatures of glioma has been furthered by studies performed within The Cancer Genome Atlas (TCGA) Network. Verhaak et al reported on the classification of GBM into Proneural, Neural, Classical, and Mesenchymal subtypes based on integrated genomic analyses. The particular transcriptional pattern and DNA copy number aberrations of EGFR, NF1, and PDGFRA/IDH1 specify the Classical, Mesenchymal, and Proneural subtypes, respectively (Verhaak et al 2010). Interestingly, while the mesenchymal subtype is defined by loss-of-function events, the other three subtypes are characterized by gain-of-function of certain oncogenes. Since the time this classification was proposed it has been clear that it is not absolute and other factors such as TME play a role in GBM biology. Namely, certain genetic changes cluster mainly within a given subclass, such as IDH1 mutations in the proneural subclass, while others like EGFR (epidermal growth factor receptor) amplification, although prevalent in the classical subtype, do occur in other subclasses as well. Technological progress has allowed for multimodal data acquisition and analysis. Based on TCGA dataset, studies are emerging where not only cell intrinsic (genetic, epigenetic and transcriptional) factors are taken into account but broader aspects of tumor pathology are included in the hope of
solving the puzzle of GBM heterogeneity (Orr and Eberhart 2012). Cooper et al focused on necrosis and angiogenesis due to the prognostic value of these properties. The authors conclude that a high degree of necrosis correlates with mesenchymal subtype and poor prognosis whereas non-mesenchymal tumors were shown to resemble the transcription profile of mesenchymal subtype with increasing level of necrosis (Cooper et al 2012). At least two concerns raised by the authors would need to be addressed before molecular subclassification could to be routinely adopted in the clinic. Firstly, a tumor sample taken from the necrotic core would ‘have’ a mesenchymal signature while other tumor areas might not. Secondly, targeted treatment of tumor with e.g. bevacizumab may enhance necrosis and hypoxia thereby changing the original transcriptional profile of the tumor at diagnosis. These examples illustrate a degree of plasticity between different subtypes (Orr and Eberhart 2012).

Epigenetic changes in GBM

DNA methylation is a heritable epigenetic event that impacts gene expression without causing changes in the DNA sequence. Epigenetic control is mediated not only by DNA methylation but also includes histone modifications leading to changes of nucleosome structure. Functionally significant epigenetic alterations are hallmarks of human cancers manifested by global DNA hypomethylation and hypermethylation of promoter CpG islands of tumor suppressor genes (Jones and Baylin 2007).

Several studies have attempted to elucidate GBM-specific epigenetic changes both on the level of the whole epigenome as well as of particular key genes (Hegi et al 2008, Kim et al 2006b). Lately, Noushmehr and colleagues carried out a comprehensive epigenome study based on TCGA cohort (Noushmehr et al 2010). They characterized the DNA methylation profile by defining a glioma-specific CpG island methylator phenotype (G-CIMP) similar to the CIMP described for colorectal cancer (Toyota et al 1999). In this phenotype, a pattern for a subset of genes in a subset of tumors emerges to further illustrate GBM heterogeneity. Interestingly, a majority of G-CIMP (cluster 1) tumors fall into the proneural subtype of GBM, whereas only 30% of proneural tumors are G-CIMP positive, further refining this subtype. Clusters 2 and 3 were somewhat enriched for classical and mesenchymal subtypes, respectively. When exploiting the clinical data associated with samples within TCGA cohort, the authors were able to correlate G-CIMP status with better prognosis, prevalence among low-grade gliomas, younger age of patients at diagnosis, and somatic isocitrate dehydrogenase 1 (IDH1) mutations. The latter yielded an amino acid substitution in an active site of an enzyme responsible for the conversion of isocitrate into α-ketoglutarate, one of the irreversible steps in the Krebs cycle. Turcan et al
have furthered our understanding of IDH1 mutations and G-CIMP interdependence demonstrating that mutant IDH1 was sufficient to alter the epigenome by progressively remodeling the global methylation landscape. The authors thereby confirmed an earlier observation that IDH1 mutations are more typical for secondary as compared to primary GBMs and demonstrate that IDH1 mutation reprograms the epigenome by interfering with the differentiation program (Turcan et al 2012).

Besides adding a layer of complexity, epigenetics reveal another aspect of GBM heterogeneity. For example, O6-methylguanine-DNA methyltransferase (MGMT) promoter hypermethylation and subsequent gene silencing, although associated with better prognosis and response to alkylating agents, can occur in distinct areas of GBM tumor surrounded by cancer cells with detectable levels of the enzyme. This makes it difficult to design universal therapeutic strategies (Hegi et al 2008).

### Alterations in glioma signaling pathways

In a multicellular organism cells depend on molecular cues that exert their effect in an endo-, para- juxta- or autocrine manner and are responsible for regulating cell faith, survival, migration etc. Signal transduction may lead to activation or alteration of function of a preexisting protein, or result in changes of the expression levels of target gene(s). G protein coupled receptors signal in the former, more rapid manner to regulate among other processes cytoskeleton rearrangements. Signaling downstream of receptor tyrosine kinases (RTKs) primarily modulates gene expression by activating transcription factors and targeting them to the nucleus. A number of second messengers convey and amplify these signals, e.g. cAMP and Ca^{2+} (Lodish 2008). Additionally, microRNAs have evolved as a class of regulators acting on post-transcriptional level to modulate mRNA expression pattern. All of these normal signaling pathways that converge in a cell can be dysregulated in gliomas.

The signaling networks mentioned above describe the complexity of ECM – host – cancer interactions. However, the diversity of glioma intracellular signaling is equally complex and heterogeneous.

Advances in microarray and sequencing technologies have enabled comprehensive genomic analysis and identification of core pathways in glioblastoma. Although suppressor genes like p53 and RB and their pathways are altered in 87% and 78% of cases, respectively (TCGA 2008), clearly making them critical players in gliomagenesis and tumor progression, they lie outside the scope of the current investigation. For recent reviews, see (Huse and Holland 2010, Zhu et al 2012). Signaling pathways, regulatory mechanisms and cytokines important for the present study are described below.
RTKs in glioma

Genetic changes in the RTK/RAS/PI(3)K network constitute a large fraction of all mutations known in glioma. Combined, these signaling pathways are altered in 88% of cases (TCGA 2008). Upon ligand binding, dimerization of receptors occurs, bringing their intracellular domains into proximity. This leads to an autophosphorylation, a recruitment of scaffold proteins and an activation of a signaling cascade. The epidermal growth factor receptor gene (EGFR) is commonly amplified or mutated (TCGA 2008) (Figure 2). A truncated version of the gene, the prevailing mutation of this receptor (EGFRvIII), encodes a constitutively active receptor that signals downstream even in the absence of ligand binding (Gan et al 2009). The EGFR pathway contributes to proliferative cues to cancer cells which translate into tumor progression and recurrence as well as poor patient prognosis. ERBB2 (Human Epidermal growth factor Receptor 2) is considered to be an orphan receptor with an unknown activating ligand (Olayioye 2001) and is mutated in 8% of GBM cases (TCGA 2008).

The presence of auto- and paracrine signaling loops within the PDGF-stimulated pathway was originally shown by our laboratory (Nister et al 1988). This notion is explained by the detection of both mRNA and protein of PDGF ligands and receptors in human glioma tissue (Hermanson et al 1992). Furthermore, this finding was successfully utilized in generating a relevant mouse model of the disease (Uhrbom et al 1998).

Although the generation of genomic profiles has aimed to address intratumoral heterogeneity, it has become increasingly obvious that the value of such profiles is limited, i.e., characteristics of the prevalent cell type within the tumor bulk are not necessarily predictive of the properties of the heterogeneous population.

Figure 2. Schematic summary of RTK pathways and incidence of alterations, adapted from (TCGA 2008). a is based on (Meng et al 2007).
An intriguing example of such heterogeneity has recently been published by Snuderl et al using fluorescence in situ hybridization (FISH) to detect copy number changes of three commonly amplified RTKs. Their work revealed that although tumor cells share a common early genetic change, they later acquire either \textit{EGFR} or \textit{PDGFRA} amplification, these events being mutually exclusive in the same clone. Furthermore, the distribution of \textit{EGFR} and \textit{PDGFRA}-amplified clones within the brain varies: unlike \textit{PDGFRA}-amplified clones, \textit{EGFR}-amplified clones are present not only in the tumor bulk but also at the invasive edge and in a secondary lesion. The authors hypothesize that this could be an example of clonal cooperation rather than competition (Snuderl et al 2011).

**Cytokines**

Cytokines constitute a diverse group of signaling molecules that is made up of growth factors, interleukins, interferons and other polypeptides and glycoproteins. They exert their plethoric, and often opposing, functions by affecting number of processes within the TME. Previously it was believed that infiltrating immune cells are the primary source of cytokines. However, compelling evidence now suggests that tumor cells, GSC and neovasculature also secrete cytokines thereby diversifying TME communication. Although not yet investigated in GBM, MC granules could be an additional and plentiful source of chemokines in brain tumors. Among different means of regulation, the cytokine repertoire within GBM has been shown to be controlled by transcription factors like signal transducer and activator of transcription 3 (STAT3) and nuclear factor kappa B. The former is critical at mediating interferon family while the latter enhances VEGF, tumor necrosis factor \( \alpha \) and IL-8 expression. Both transcription factors can induce IL-6 secretion.

Examples of IL-6’s involvement in promoting angiogenesis, proliferation and radiotherapy resistance have been demonstrated (Zhu et al 2012). Furthermore, enhanced IL-6 signaling promotes gliomagenesis by facilitating GSC growth and survival, and is associated with poor prognosis (Wang et al 2009). I have previously described intratumoral heterogeneity in terms of differential hypermethylation of \textit{MGMT} promoter within the tumor bulk (Hegi et al 2008). Another study provides the first illustration of intratumoral heterogeneity and cooperative behavior between wild type (wt) and mutant \textit{EGFR} expressing cell populations present within one tumor. The Furnari laboratory demonstrated that ligand-independent constitutive signaling of EGFR\textit{vIII} led to induction and secretion of IL-6 and leukemia inhibitory factor, which effectively activated corresponding downstream signaling in EGFR wt cells (Inda et al 2010).

GBM is a highly vascularized tumor where angiogenesis is induced by a number of growth factors, such as PDGF, TGF-\( \beta \), VEGF, and chemokines like CXCL12 and IL-8 (Hanahan and Coussens 2012). The role of the latter
in promotion of the angiogenic process is linked to its ability to induce MMP production which, in turn, facilitates vascularization of the tumor (Li et al 2003). Physiological levels of IL-8 are almost undetectable, but in brain tumors its amount escalates in a grade-dependent manner (Zhu et al 2012).

Although not specifically investigated in the framework of this thesis, TGF-β is considered to be the most studied participant of the malignant glioma landscape. It binds to its receptor (T-βR) causing a cytoplasmic signaling cascade which leads to activation of Smad transcription factors and subsequent changes in the expression of target genes (Iwami et al 2011). Additionally, its immunosuppressive role is manifested in a shift of cytokine expression pattern, inhibition of lymphocyte proliferation and decrease in immune cell activation (Letterio and Roberts 1998).

Chemochines are a class of cytokines with chemotactic properties. Two representatives of this subfamily of cytokines important for present investigation (CXCL12 and MIF) are described below.

**CXCL12** (also known as stromal-derived factor 1) expression is grade-dependent, although unevenly distributed within GBM where it is more pronounced in the regions of angiogenesis and necrosis (Rempel et al 2000). Inhibition of this chemokine and its receptor delays tumor recurrence by blocking recruitment of macrophages and tumor revascularization (Tseng et al 2011). Besides these well-established effects on TME, CXCL12 it has been shown to promote glioma cell (Barbero et al 2002) and GSC (Ehtesham et al 2009) proliferation.

**MIF** expression is known to be elevated in a number of immunoedited cancers including glioblastoma where its presence is associated with worse prognosis, recurrence, and high expression of IL-8. This may be due to its antiapoptotic and proangiogenic activities (Wang et al 2012). Its role in a cancerous ecosystem has also been demonstrated in medulloblastoma where tumor cells secrete MIF that induces vascular cell secretion of RANTES which in turn recruits T lymphocytes (Salsman et al 2011).

**CXCR4** is the receptor for both CXCL12 and MIF, and we have recently demonstrated that both of these glioma-expressed chemokines are capable of recruiting MCs in vitro (Paper I and II, respectively).

### Rap1 signaling and implications in cancer

Guanosine triphosphate (GTP)-binding proteins convey intracellular signals and are implicated in a number of cellular processes, including cell growth and differentiation. Heterotrimeric (“large”) G proteins contain 3 subunits, whereas monomeric (“small”) G proteins have only one. The latter make up the Ras superfamily of G proteins and include Rap1. G proteins act as allosteric switches, similar to the ATP/ADP switch, by hydrolyzing GTP to GDP (Lodish 2008).
Rap1 is active when GTP-bound and the time during which it remains active is determined by the rate of GTP hydrolysis. Rap1 has low intrinsic GTPase activity, but multiple GTPase activating proteins (GAPs) enhance this process. Guanine exchange factors (GEFs) facilitate Rap1 activation by causing release of GDP and binding of intracellularly abundant GTP (Bos et al 2001). Commonly studied mutations within the Rap1 gene include constitutively active and dominant negative variants with amino acid substitutions in positions 12 (Gly to Val) and 17 (Ser to Asn), respectively (Kitayama et al 1990). Rap1\textsuperscript{CA} remains in a GTP-bound form as it is resistant to the activity of GAPs. On the other hand, Rap1\textsuperscript{DN} forms unproductive bonds with GEF thereby titrating out the wild-type Rap1. In an experimental set up, Rap1\textsuperscript{DN} and Rap1GAP are interchangeable and have identical effects on the cell.

Rap1 was identified as a GTPase able to revert K-ras-mediated transformation (Kitayama et al 1989) but is now implicated in a number of processes unrelated to cancer, such as neuronal development. Overexpression of Rap1GAP and subsequent inactivation of Rap1 leads to an enlarged cell body of medium spiny neurons (McAvoy et al 2009). Rap1GAP also negatively impacts glial cell line-derived neurotropic factor-mediated neurite outgrowth (Jiao et al 2011). However, despite reverting astrocyte proliferation caused by constitutively active Ras, Rap1 had no effect on the number of astrocytes in wild-type mice as compared to constitutively active Rap1 expressing transgenic littermates (Apicelli et al 2003).

The function of Rap1 is largely cell type specific and is tightly regulated in time and space. Rap1 exists in two isoforms, Rap1a and Rap1b, differing in just 9 amino acids which poses experimental obstacles in distinguishing between them (Frische and Zwartkruis 2010). Often the functions of both isoforms are redundant but isoform-specific signaling has been described. For example, PDGF-BB stimulates smooth muscle cell growth by upregulating Rap1\textsuperscript{a} but not Rap1\textsuperscript{b} mRNA species (Quarck et al 1994). The variation of the effect Rap1 has on a cellular process can be explained by the Rap1 effectors’ availability in specific cell context.

Deeper understanding of the role of Rap1 in mammalian physiology was obtained from knockout mice. Rap1a-/- mouse strains are viable, fertile and immunocompetent (Duchniewicz et al 2006, Li et al 2007). However, researchers have reported a number of abnormalities in the adhesion of cells of the hematopoietic lineage: macrophages adhered poorly on fibro- and vitronectin and exhibited an increased rate of random migration; directional migration of both lymphoid and myeloid cells was decreased. These data manifest a connection between Rap1a and integrin activation.

Indeed, the regulation of inside-out integrin signaling by Rap1 has been demonstrated in a number of cell types. In summary, Rap1 signals to cytoskeleton-associated (but not to intermediate filament related) integrins (Bos et al 2001). Takahashi et al demonstrated that activation of Rap1 is required for PDGF-induced cell movement (Takahashi et al 2008). In the
field of hematology, the ability of hematopoietic cells to migrate and home to target tissues is key to their functionality. Rap1’s contributions have proven to be critical in this regard (Stork and Dillon 2005). Activation of Rap1 leads to increased adhesion and polarization of monocytes (Lorenowicz et al 2006).

Rap1 also plays a role in cadherin-mediated cell adhesion as disruption of Rap1 signaling leads to loss of epithelial cell-cell contacts (Price et al 2004) and uneven distribution of Drosophila E-cadherin in the cells of the wing (Knox and Brown 2002). This effect may be caused by changes in the endocytic recycling of cadherin.

It has been increasingly recognized that anchorage conveys important signals for stemness maintenance. Although Rap1 does not directly alter expression of pluripotency genes like Oct4 and Nanog, it instead maintains E-cadherin-mediated cell-cell cohesion, which is essential for human embryonic stem cell self-renewal (Li et al 2010). These data are in accordance with findings from Lasorella’s laboratory who implicated Rap1 signaling in neural stem cell (NSC) adhesion and stemness maintenance. Id proteins orchestrate residency of NSC in the neural niche by maintaining Rap1 in active, GTP bound state. Id gene deletion resulted in increased Rap1GAP levels. Also in a given experimental setting, Rap1GAP signaling did not alter the differentiation state of the cells per se but was instrumental in disrupting cell adhesion which is critical in NSC stemness maintenance (Niola et al 2012). This mechanism seems to be evolutionarily preserved as Rap1 signaling in concert with E-cadherin regulates stem cell anchoring in Drosophila testis (Wang et al 2006).

There is extensive evidence that Rap1 exerts its function in cancer cell migration and invasion by activating critical integrins. Most recently, the importance of Rap1 for integrin function has been demonstrated in colon (Tsygankova et al 2010), melanoma (Zheng et al 2009), prostate (Bailey et al 2009) and pancreatic (Ricono et al 2009) cancers. Furthermore, in the latter publication, the authors describe the necessity of cross-talk between EGFR and integrin αvβ5 for carcinoma cell invasion and metastatic expansion.
MicroRNAs

Biogenesis

RNAs can be divided into two classes: coding RNAs, i.e., mRNAs and non-coding species including among others transfer, ribosomal, small interfering (siRNAs) and microRNAs (miRNAs). The two latter classes have been known for less than 20 years (Hamilton and Baulcombe 1999, Lee et al 1993). Because mature si- and miRNAs are only approximately 20 nucleotides long, their discovery has been hindered due to the lack of suitable experimental techniques and a long-standing belief that there are a limited variety of non-coding RNAs. To date 2019 miRNAs have been annotated in humans (www.mirbase.org, Database Release 19.0). The complexity of higher organisms might originate from the large fraction of non-protein-coding sequences in the genome (98.5% in humans) (Mattick 2004). MicroRNAs represent a novel class of gene regulators, affecting more than a third of coding mRNAs (Lewis et al 2005).

Mature si- and miRNAs are similar in size, regulatory function and biogenesis differing only in details of their mechanism of action. Namely, siRNAs exhibit full complementarity to their target that leads to cleavage of the respective mRNA. On the contrary, miRNAs only partially match the sequence of mRNA in question, resulting in an inhibition of translation (Bartel 2004). The majority of miR genes are transcribed by RNA polymerase II (Lee et al 2004). They are thereafter spliced and polyadenylated, generating a pri-miRNA transcript. The latter is further processed by enzymes of the nuclear ribonuclease family, Drosha and Dicer, generating precursor microRNA (pre-miRNA). This up to 100-base long RNA has a hairpin structure and is exported into the cytoplasm by exportin 5. There, microRNA completes its maturation with the help of Dicer and Loquacious. The passenger strand is separated from the guide strand and the latter is loaded onto an RNA-induced silencing complex (RISC) to provide sequence-specificity in mRNA targeting (Kosik 2006).
Deregulation in GBM

Rapid development of sequencing and microarray technology has facilitated microRNA (miR for short) profiling in various cancer types, including GBM. An expression signature of ten microRNAs has been demonstrated to predict patient survival where three miRs are thought to play a protective role while expression of the remaining seven miRs is associated with higher risk (Srinivasan et al 2011). Tumor progression is associated with changes in the genomic and epigenomic landscapes. Expectedly, miRnome is also altered in a grade-dependent manner. In particular, the expression of microRNAs -21, 196a, -196b and 156 was shown to dramatically increase throughout glioma progression. Furthermore, expression of miR-21 and miR-34 was significantly lower in secondary as compared to primary GBMs providing further evidence for a difference in molecular signature between these two groups of GBM (Ma et al 2012). TCGA subtype-specific miR signatures are beginning to emerge with 20 G-CIMP specific microRNAs being one of the early examples (Noushmehr et al 2010).

There are large numbers of potential miRNA-mRNA matches generated in silico. These computational algorithms predict complementarity between the 3’UTR sequences of mRNAs that are preferentially conserved between species and positions 2-8 (termed seed region) of a particular mature miRNA (Okamura et al 2008). However, only a fraction of these predictions have been verified in vitro. As summarized by Di Leva et al, consecutive studies in GBM focusing on elucidating specific mRNA targets have demonstrated that miR-9 is upregulated in GSC and targets a tumor suppression gene, CAMTA. Furthermore, miR-17/92 cluster promotes angiogenesis by altering TGFβ signaling. Finally, cells acquire high levels of miR-221/222 which promotes gliomagenesis by regulating apoptosis and cell proliferation (Di Leva et al 2012).

miR-21: an oncomiR to die for

Since the original discovery that miR-21 acts as an apoptotic factor in glioblastoma cells (Chan et al 2005), this microRNA has been shown to be upregulated in virtually all cancer types tested illustrating its primary role as an oncomiR.

Performing siRNA transfections followed by real-time PCR, Papagiannakopoulos et al were able to identify a number of miR-21 targets in glioblastoma cells. The generated list of genes was subjected to the Ingenuity Pathway Analysis (www.ingenuity.com) that grouped the targets based on their affiliation with a specific pathway. In concordance with in silico predictions, identified targets mapped to p53, TGF-β and mitochondrial apoptosis path-
ways (Papagiannakopoulos et al 2008). Another independent study experimentally confirmed PTEN (phosphatase and tensin homolog) to be a target of miR-21 in hepatocellular carcinoma cells (Meng et al 2007).

The bulk of the data described above have been generated using cell cultures, tissue samples and a limited number of xenografts. A more systematic approach in the form of transgenic mice studies deepens our understanding of miR-21 biology. In 2010 Hatley et al reported the first transgenic mouse with loss-of-function and gain-of-function miR-21 alleles (Hatley et al 2010). Using these mice in their lung cancer model they demonstrated that miR-21 plays a role in tumor development by inhibiting apoptosis and through a positive impact on the Ras/MEK/ERK pathway. Additionally, Medina et al defined microRNA-21 as an oncomiR addiction causing gene in their in vivo model of pre-B-cell lymphoma. The authors demonstrated a complete regression of tumors following miR-21 inactivation (Medina et al 2010). In a skin carcinogenesis model, miR-21-null mice developed significantly less papillae as compared with wild-type mice (Ma et al 2011).

In recent years the focus has shifted from identifying various microRNA targets to identifying key players in microRNA regulation. This field of investigation is especially intriguing in the case of miR-21, since it is commonly upregulated in cancer. Researchers have discovered a positive regulatory effect of IL-6 in multiple myeloma (Loffler et al 2007), TGF-β in breast cancer (Qian et al 2009) and EGF in lung cancer (Seike et al 2009) on miR-21 levels. As expected, BMP-6 inhibits miR-21 expression in breast cancer (Du et al 2009). In 2012, the first example of GBM-specific miR-21 regulation was published. Han and colleagues indicated that an upstream miR-21 promoter contained a conserved STAT3 binding site and was therefore regulated by β-catenin in a STAT3-dependent manner (Han et al 2012a).

During the year 2012 we saw an avalanche of publications where circulating miR-21 detectable in serum was coined as a marker of various solid tumors, e.g. breast, esophageal, gastric, colorectal and lung cancers (Teplyuk et al 2012, Wang and Zhang 2012). In cerebrospinal fluid, levels of miR-10 alongside miR-21 were significantly increased in patients with GBM and brain metastasis of lung and breast cancer as compared to patients with non-neoplastic disorders of CNS (Teplyuk et al 2012). Furthermore, the value of miR-21 as a prognostic marker has been highlighted in studies of colon adenocarcinoma (Schetter et al 2008) and astrocytoma (Zhi et al 2010) where high levels of this oncomiR are consistently associated with poor prognosis.

miR-21 has been implicated in all fundamental characteristics of GBM biology, i.e. proliferation (Papagiannakopoulos et al 2008), invasion (Han et al 2012a), and drug resistance (Wong et al 2012). Our work indicates the existence of developmental regulation of miR-21 and its importance in sustaining SOX2 expression in glial progenitor/stem cell type (Paper IV). The hypothetical importance of miR-21 in the maintenance of GSCs and GBM-favorable microenvironment remains to be elucidated; however examples
from other cancers indicate that it very well may be the case. In particular, inhibition of miR-21 reverses cancer stem cell phenotype in breast cancer (Han et al 2012b) whereas tumor secreted miR-21 mediates a prometastatic inflammatory response in lung cancer (Fabbri et al 2012).
At the moment when a patient is presented to the clinic, the doctor is often faced with a full-blown disease. Based on post-surgery samples alone it is impossible to study tumor initiation and progression events. When it comes to recapitulating the chronology of mutations, mouse models have been most instrumental. Nowadays, a plethora of genetically engineered mouse models are available, which facilitate the study e.g. sub-type specific events and the impact of TME. However, modeling heterogeneity of GBM fully remains a challenge. In xenotransplantation models, in vitro artifacts are minimized by using primary patient-derived material (Bonavia et al 2011, Huse and Holland 2010).

High-throughput sequencing is a powerful and essential tool for identifying common mutations in the glioma genome but does not discriminate between passenger versus driver mutations. Insertional mutagenesis provides an important technique for the discovery of genes important for glioma initiation.

Retroviral mutagenesis has been successfully utilized to tag genes in a number of hematopoietic as well as solid tumors. In a pioneering study, Johansson et al combined a transforming murine retrovirus encoding PDGF-B with high-throughput sequencing to generate a list of 66 brain tumor loci (Btl) potentially important for gliomagenesis (Johansson et al 2004). Members of our group have successfully investigated in great detail the functional role of a number of candidate glioma genes from some of the identified Btls (Ferletta et al 2007, Singh et al 2009, Swartling et al 2009, Tchougounova et al 2009, Westermark et al 2011). In the present investigation, we aimed at unraveling the role of two of the tagged brain tumor genes, namely miR-21 (Btl-12) and Rap1gap. The former was tagged twice and the latter three times. Notably, at the moment when the study of Johansson et al was conducted, the miR-21 structure and localization had barely begun to be unraveled. At present, researchers acknowledge the importance of this oncomiR in a number of cancers, as described above.
**Therapy**

“Looking for a cure is not the same as prolonging a life”

*Eric C. Holland at EACR-22, 2012*

As a rule, all GBM patients undergo surgery followed by a combination of radio- and chemotherapy. Due to the documented effect temozolomide (TMZ) had on patient survival; this drug is now concomitantly used with ionizing radiation. This orally administered agent acts both by alkylating DNA and by exhausting the active pool of DNA-repair enzyme MGMT (Tolcher et al 2003). The response of patients to TMZ is particularly associated with hypermethylation of *MGMT* promoter (Hegi et al 2008).

As of now the EGFR inhibitor Gefitinib (Iressa®) has had a marginal effect on the overall survival of patients with recurrent glioma enrolled in Phase II clinical trials (Rich et al 2004). Furthermore, despite successful applications in other cancer types, the effect of imatinib mesilate (Gleevec®; an inhibitor of PDGFR, KIT and ABL) was limited to a subset of patients (Desjardins et al 2007). However, Gleevec has the ability to lower interstitial fluid pressure which could be utilized to make drug delivery more efficient (Pietras et al 2001). As GBMs are highly vascularized, they have been expected to respond remarkably to inhibition of angiogenesis. Nonetheless, usage of humanized monoclonal antibodies against VEGF (Bevacizumab, Avastin®) and small-molecule inhibitors against a number of VEGF receptors has had disappointing results. The majority of patients developed resistance and in some cases alternative proangiogenic pathways (via e.g. basic fibroblast growth factor and CXCL12) were activated (Arbab 2012). A better understanding of the effect of VEGF inhibition on the perivascular niche holds great potential, as this area is known to have an intimate relationship with GSC.

Since GSCs are thought to be the major culprits of GBM recurrence and therapy resistance, a lot of efforts are being made to selectively target them. In a randomized controlled phase IIb trial, brain tumor patients intratumorally receiving traberersen (AP-12009, a synthetic antisense oligodeoxynucleotide blocking TGF-β2 production) had higher survival rates as compared to patients receiving chemotherapy alone (Vallieres 2009). This could be due in part to the importance of TGF-β2 signaling in GSC invasion (Reardon et al 2009) as well as stemness maintenance (Ikushima et al 2009).
CXCR4 (receptor for CXCL12 and MIF) is overexpressed in GSC as well as in invading GBM cells. Antagonists of this receptor are being tested (Zhu et al 2012) and could potentially also be used to inhibit recruitment of MCs to the tumor although it is still unknown whether MCs facilitate or impede gliomagenesis.

Besides the vasculature, attempts to target other components of TME have been made. Attempts are made to target the cytokine network by gene therapy and antibodies. Activation of cytotoxic T cells is also attempted (Iwami et al 2011, Zhu et al 2012).

The possibility of targeting microRNAs as a part of anti-cancer therapy seems worthy of a try for a number of reasons. Firstly, a single microRNA may target a repertoire of cancer-associated genes. Secondly, the development of modified miRNA molecules increases their stability and half life further improving their efficacy (Calin and Croce 2006). Lastly, delivery of large quantities of miRNA may be carried out with the help of liposomal agents (Esquela-Kerscher and Slack 2006). miR-21 is especially an excellent target. Its expression is high in the tumor and absent in adult brain (Paper IV), its inhibition causes apoptosis and sensitizes cells to both upfront and acquired TMZ resistance (Shi et al 2010, Wong et al 2012).

The blood brain barrier is a major hurdle in GBM treatment. It is compromised within the tumor causing edema and hindering efficient drug penetration. However, the BBB is intact in the parenchyma where the scattered cancer cells reside. As exemplified above, in addition to systemic drug delivery, researchers have attempted to use gene therapy, direct intracranial injections and osmotic pumps to ensure that the agents reach their destination.

Another pharmacological challenge is the intra- and intertumoral heterogeneity described above. Although sequencing of the glioma genome addresses the intertumoral differences, cell-cell variations continue to pose a methodological challenge. Nevertheless, increasingly detailed understanding of GBM subtypes is bound to result in mechanism-guided treatment, which holds great potential in improving clinical outcome.

As described above, heterogeneity is observed on every level. Resistance to treatment and lethality are caused by heterogeneity. This aberrant ecosystem is diverse, full of compensatory mechanisms and when selective pressure is applied, new aspects of its heterogeneity and adaptability are revealed.
Present investigation

Aims
The overall goal of the work presented in this thesis was to gain a deeper understanding of the immunological landscape of GBM, its invasive nature and its addiction to oncomiR-21. Specific goals for each paper were as follows:

I To establish whether MCs are present in experimental and human glioma, and to elucidate a potential chemotactic axis for glioma-MC interaction.
II To study grade-dependent accumulation of MCs in glioma, to elucidate the GBM cytokinome, to correlate the expression of discovered candidates with MC accumulation and to study the effect of MC-GBM cells cross talk on invasive properties of the latter.
III To study the potential involvement of RAP1GAP in various aspects of GBM biology and the underlying mechanisms involved.
IV To elucidate the role of microRNA-21 in embryonic development of the brain and tumor self-renewal in PDGF-driven experimental glioma in cooperation with SOX2

Results and discussion

Paper I: Mast Cell Accumulation in Glioblastoma with a Potential Role for Stem Cell Factor and Chemokine CXCL12

Although known for their involvement in allergic reactions and opposing roles in different cancer types, MCs have, until recently, not been described in glioma.

We first focused our attention on experimental GBM, demonstrating a striking increase in MC numbers in Arf knockout tumor bearing mice. MCs reside in the brain in an area called the medial habenula which could be the source of MCs in the tumor. Alternatively, MC recruitment could occur from the periphery. Roughly half of MCs were localized in the vicinity of tumor vessels that expressed significantly higher levels of SCF as compared to
vessels outside the tumor margin. SCF is an essential stimulant of MC proliferation and maturation. We therefore hypothesized whether local plentiful supply of SCF could also expand MC population in situ and verified this by confirming that tumor associated MCs express Ki67, a marker of proliferation.

The chemokine signaling network within GBM is diverse. We reasoned that besides SCF/KIT axis, other ways of MC recruitment must exist and investigated CXCL12 due its known chemotactic properties and increased expression in malignant tissues. Immunofluorescence imaging revealed a significant increase of CXCL12 expression in the tumor area as compared to non-tumor controls. Besides the tumor mass, endothelial cells contributed to CXCL12 pool (Figure 3). In addition, a vast majority of MCs were positive for CXCL12 receptor, CXCR4. The biological relevance of these observations was further confirmed by mechanistic trans-well assay in which neutralization of either the ligand or the receptor led to marked decrease in MC migration.

Subsequently, we turned our attention to the human condition and validated the existence of CXCL12/CXCR4 signaling axis in patient samples.

Figure 3. MC recruitment to GBM is facilitated by a gradient of different chemokines that are secreted by endothelial cells (EC), GBM cells and glioma stem cells (GSC), based on Paper I and II.

Paper II: Mast Cells Are Recruited to Glioma and Orchestrate Cancer Cell Invasion

In Paper I grade-dependent accumulation of MCs in glioma was established on a limited patient sample set (n=18). In Paper II, MC accumulation in relation to tumor grade was investigated on a large sample set (n=184) using tissue microarray (TMA) reconfirming our initial findings.

For mechanistic studies, two cell lines were used, both established in our laboratory using different culturing conditions. U-2987 MG has been grown...
in serum-containing Minimal Essential Medium whereas the low passage cell line U-3054 MG has been grown under serum-free conditions known to enrich for GSCs. Supernatants from both cell lines were able to significantly induce trans-well migration of MCs. A systematic approach in determining the cytokinome of these cell lines was adopted, revealing an overlap in expression of some cytokines as well as a set of cell line specific candidates. MIF was secreted by both cell lines tested and was selected for subsequent investigation due to its known role in promoting angiogenesis and leukocyte recruitment. MIF expression was assessed on the same sample set as described above, revealing a heterogeneous expression pattern in terms of both cytoplasmic intensity and cytoplasmic fraction.

MIF expression pattern correlated with MC infiltration, encouraging us to investigate whether MIF acts as a chemoattractant for MCs in GBM (Figure 3). Indeed, neutralizing antibodies against MIF significantly decreased MC trans-well migration. CXCR4 is one of the receptors for MIF. Although not investigated in Paper II, our finding that MCs are positive for CXCR4 (Paper I) could be applied here to state that MIF/CXCR4 axis is another potential way for MC recruitment to GBM.

Signaling cascades in MCs as brought about by the GBM microenvironment are largely unknown. We have investigated changes in MC kinome using a phospho-kinase array. As expected, chemokines secreted by GBM cells into the medium increased the phosphorylation of a number of STATs and other kinases. Interestingly, phosphorylation of CREB in MCs exposed to GBM-conditioned medium was decreased when compared to control MCs.

Crosstalk between GBM cells and MCs was investigated in a three-dimensional gel system. MC mediators significantly induced GBM cell invasion and the exact mechanism underlying this striking effect is the focus of ongoing experiments in our laboratory.

Paper III: RAP1GAP Suppression Promotes Stemness and Inhibits Glioblastoma Cell Migration

Members of the Ras superfamily are extensively studied in health and disease, including cancer. Surprisingly little is known about the role of Rap1 in glioma. RAP1GAP, known to induce intrinsic GTPase activity of Rap1, was previously identified as a brain tumor candidate gene. We set out to investigate its role in glioma in vitro.

TCGA data confirm relevance of our investigation. Thus, RAP1GAP is prevailingly downregulated in GBM patients. The degree of this downregulation is most pronounced in the mesenchymal subtype.

U-2987 MG clones with inducible shRNA expression targeting RAP1GAP were created and validated. Proliferation was assessed by a num-
ber of methods demonstrating no sustained effect on cell growth following alterations in RAP1 signaling.

Poor survival of glioma patients is mainly associated with recurrent tumor formation that is caused by highly infiltrative cells remaining in the brain after dissection of the tumor bulk. We therefore turned our attention to elucidating the potential role of RAP1GAP in tumor cell migration. A Boyden chamber assay revealed a significant negative effect \textit{RAP1GAP} silencing had on cell migration which seems not to be dependent on cell spreading or cell adhesion.

In light of recent studies investigating the involvement of RAP1GAP in adhesion of NSC, we hypothesized that similar mechanisms may govern GSC adhesion. Investigation of SOX2 and GFAP expression after shRAP1GAP induction revealed an increase in the SOX2/GFAP double positive fraction, indicating a shift towards a stemness phenotype. A classical limited dilution assay should be optimized for U-2987 MG cells to further address this finding. Alternatively, a low-passage GSC culture could be infected with shRAP1GAP to provide better experimental conditions for studying the effects of RAP1GAP on stemness. Potential changes in levels of other stemness markers like Nestin should be determined.

Since our findings do not point to integrins as executors of activated RAP1 signaling in this model, the involvement of cadherins needs to be investigated.

To sum up, Rap1 has an effect on migration of glioma cells which seems to be coupled to stem cell phenotype. The precise mechanisms of this effect are yet to be elucidated.

\textbf{Paper IV: miRNA-21 is Developmentally Regulated in Mouse Brain and is Co-expressed with SOX2 in Glioma}

During the past decade microRNAs have been increasingly studied as a novel class of non-coding RNAs. One of the most studied microRNAs, miR-21 acts as an oncogene in a majority of cancers.

In this study we investigated the regulation of miR-21 in PDGF-B-driven glioma, its effect on stemness, and its expression pattern during normal development. Based on the recent observation that in PDGF-B-driven tumors glial progenitor/neural stem cells may act as cells of origin, we investigated the miR-21 expression pattern in normal development. We discovered that miR-21 is developmentally regulated: its pronounced expression gradually decreases from E18 onwards and almost vanishes by P7 (\textit{Figure 4}, top panel). Its expression pattern overlaps with Sox2 expression in the developing mouse brain.
miR-21 is differentially expressed in experimental tumors depending on the cell of origin and genetic background of the mice. This oncogenic microRNA is highly and specifically overexpressed in the tumor area as shown by in situ hybridization, co-localizing with Sox2 and Olig2 in the brain.

Based on the bioinformatic prediction tools available online, translation of Sox2 is expected to be negatively regulated by miR-21. Surprisingly, we found an opposite effect of miR-21 on Sox2 in our system such that the levels of Sox2 decrease upon miR-21 knock-down using an siRNA approach.

We then focused our attention on upstream regulators of miR-21. Due to low endogenous levels of miR-21, human foreskin fibroblasts were chosen for experiments addressing which growth factors regulate miR-21. We conclude that miR-21 expression can be induced by a number of cytokines and growth factors, including PDGF-BB. Knock-down of PDGF-BB in PDGF-B-driven tumors resulted in significant decrease of miR-21 levels. Furthermore, miR-21 levels were decreased following inhibition of a number of downstream effectors of RTK signaling pointing to PDGF-BB as a driver of miR-21 expression in experimental glioma (Figure 4, bottom panel).

Glioma cells seem to have an oncogenic addiction to miR-21 and hence are extremely sensitive to miR-21 knock-down as detected by presence of cleaved caspase-3 and Annexin-V staining. These findings are in agreement with previously published reports. The role of miR-21 we observed in the experimental mouse glioma is applicable to the classical as well as primary glioma cells lines of human origin.
Conclusions and future perspectives

As outlined in the introduction, GBM is truly an ecosystem with its ‘inhabitants’ in continuous communication with each other. With the present investigation we have highlighted some aspects of GBM biology.

As of now, GBM is an incurable disease and patients rarely live longer than 2 years after diagnosis despite intensive therapy. At the time of diagnosis a number of glioma cells have infiltrated the healthy tissue making complete resection impossible. There is a need for non-invasive early diagnostic markers. miR-21 has been found in cerebral spinal fluid of GBM patients and its diagnostic value has been demonstrated in other cancer types making it a great candidate for prognostic and diagnostic testing.

Currently, the WHO grading system is used for glioma diagnosis in the clinic. Two aspects of tumor microenvironment – necrosis and vascular proliferation- are part of grading scheme. Whole-genome sequencing has enabled mapping of the GBM genome, including changes in gene copy numbers and mutations. Division of GBM genetic alterations into 4 subtypes is not yet routinely applied in the clinic but is emerging as an important tool for assigning patients into appropriate clinical trials. Usage of standard care has reached a plateau in terms of survival. Also, the adjuvant therapies tested are introduced as add-ons increasing both costs and toxicity. Determining the molecular profile of the tumor would therefore be a cost-effective way to design personalized therapy schemes.

Tumor microenvironment has emerged as an intense field of study not only in GBM but in cancer research on the whole. In the two first papers of this thesis, MCs and their interaction with GBM are under the spotlight. This field of study is just emerging as we were first to demonstrate presence of MC in glioma in 2011 (Paper I). By now a number of MC recruitment mechanisms have been indentified and the focus is shifting to effects of MCs, more specifically their mediators, on different aspects of GBM biology, e.g. proliferation, migration and vascularization. Some of these aspects could be partially studied in vitro, but MC knockout models are irreplaceable and valuable tools for determining, e.g. MC effect on overall tumorigenicity or incidence.

Another ‘hot’ topic in glioma research during the past decade has been the role and characteristics of GSC. The terminology remains obscure and it seems to be a matter of definition whether a cell is termed a cell of origin, a glioma initiating cell or a cancer stem cell. Time will tell whether these terms are merely different facets of the same feature or represent distinct cell populations. Clear definition of characteristics of heterogeneous cell type present on the GBM landscape will further aid design of specific treatment regiments.
Poor survival of GBM patients is associated with the recurrence of treatment resistant tumors. There are indications that miR-21 contributes to the acquisition of temozolomide resistance in glioma cells. Since it is highly expressed in the tumor tissue as opposed to normal brain, miR-21 is an excellent therapeutic target. The beauty of microRNA biology is that microRNAs are pharmaceutically targetable which translates into great potential in applying targeted drugs in combination with classical treatment to improve patient survival.

Although GBMs are confined to the tissue of origin, their invasive nature poses a great challenge for surgeons. In this thesis aspects of invasiveness have been investigated from the perspective of RAP1GAP. Initially found in our screen for brain tumor candidate genes, RAP1GAP is down-regulated in essentially all samples in TCGA dataset. In Paper III we demonstrate an effect of RAP1GAP silencing on GBM cell migration and suggest a role in stemness. We are at a stage of the project where we clearly see the missing pieces of the puzzle. Is the effect on cell migration mediated by cadherin signaling? Activation of RAP1GAP promotes NSC detachment and differentiation. Are these observations applicable to GSCs and if so, can RAP1 be therapeutically targeted thereby exhausting the pool of recurrence-causing GSCs? U-2987 MG clones established in this study have been stereotactically injected into immuno-compromised mice and we eagerly await results from this in vivo experiment and plan to address the questions raised above in the near future.

In conclusion, our studies have furthered our understanding of GBM as a heterogeneous ecosystem. Some of the molecules investigated would be perfect candidates for therapeutic targeting which aims at prolonging patient survival in a meaningful way.
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