Chapter 1

General introduction and scope of the thesis
General introduction

Glioblastoma

Glioblastoma (GBM) is a highly malignant brain tumor that still today leaves diagnosed patients with a universal grim prognosis. GBM accounts for approximately 55% of all primary brain and CNS malignancies in the US population, and the yearly incidence from 2009 to 2013 was reported to be 3.2 new cases per 100,000 population [1]. The malignancy tends to affect males with a higher frequency than females (1.6 times more common) and incidence rates increase with age.

GBMs can arise as primary or secondary tumors [2]. A primary or de novo GBM concerns a tumor that has arisen spontaneously and clinically or histologically no evidence can be collected for a less malignant precursor lesion. When such evidence for a less malignant precursor lesion in the brain is found and the disease process resembles gradual progression through disease severity stages, the tumor is considered to be secondary. At the molecular level mutations in the isocitrate dehydrogenase 1 (IDH1) gene are strongly associated with lower grade gliomas [3], and GBMs carrying mutations in this gene are nowadays considered to be secondary GBMs.

At this time, however, all patients are treated uniformly, regardless of a GBM being diagnosed as a primary or secondary malignancy. The current standard of care in the first-line setting comprises of maximal safe surgical resection and chemo-radiation [4]. Although the addition of the chemotherapeutic Temozolomide over a decade ago reflects the most recent improvement of GBM prognosis [5,6], the extent of surgical resection remains to be the strongest predictor of patient survival [7-9]. The highly infiltrative nature of GBMs will however continue to prevent total resections and thus additional therapies are warranted for this malignancy [10].

There is more variety in the therapeutic approach in the recurrent setting with very few effective treatment options available. When a tumor regrows re-operation on occasion is feasible, and lastly newer and often experimental drugs can be considered.

Molecular heterogeneity

An initiative to enhance the understanding of the complicated and heterogeneous disease GBM was to analyze the genome-wide transcriptional profiles of these tumors. Rationale for this approach was to identify key molecular drivers that define subcategories of GBMs that could ultimately be addressed therapeutically. Several groups have independently reported on potential transcriptional molecular subclasses in GBM [11-13], of which the proneural (PN), classical (CLAS) and mesenchymal (MES) subclasses have been described most consistently and are assessed in the majority of follow-up research. Each of these subclasses has been associated with specific molecular aberrations, and differences in overall patient
survival and response to therapy have been reported as well [12,13]. Several ongoing clinical trials have adopted molecular aberrations in their inclusion or exclusion criteria to identify patients that could selectively benefit from the drug under study.

**Angiogenesis**

Within a GBM several non-tumor modalities contribute to and influence disease progression, and due to the high level of angiogenesis in GBM therapeutic exploration of this modality is highly sought after. Microvascular proliferation is a hallmark criterium for the diagnosis GBM by the pathologist, and this malignancy is amongst the most vascularized solid human cancers [14-16]. The high expression of angiogenic factors and especially VEGFA in high-grade astrocytomas was already observed over two decades ago [17], and currently VEGFA inhibition is clinically approved for recurrent GBM. More recent experimental work is advancing insight into alternately involved angiogenic factors, and combinations of diverse anti-angiogenic drugs is are also being explored both in the laboratory as well as in the clinical setting [18,19].

**Scope of the thesis**

The work described in this thesis was aimed at the assessment of the differential molecular characteristics and functional responses of GBMs with dissimilar phenotypes. The characteristics of transcriptional subclasses were studied in detail through the definition of molecular subclasses at protein level and the assessment of kinase activity profiles and vascularization patterns. In addition, the plasticity or interchangeability of these molecular phenotypes in response to microenvironmental cues was assessed through *in vitro* and *in vivo* GBM models. The second part of the thesis then focuses on the role of angiogenesis in GBM in general, and we have also explored the therapeutic potential of intervention in this process.

The first part of the thesis thus comprises a selection of chapters focused on the molecular subclasses in GBM, and this part is commenced by a review in Chapter 2 that outlines the effect of important microenvironmental cues on the properties of glioma stem-like cells (GSCs). Particularly GSCs are important therapeutic targets, because they are resistant against current standard therapies (chemo-radiation) and possess the ability to re-initiate tumor growth [20-22]. They are therefore utilized for *in vitro* and *in vivo* experiments in many experimental studies, and the subclass plasticity was therefore in this thesis also assessed in GBM cells with stem-like characteristics.

**Chapter 3** describes the exploration of the translational potential of the transcriptionally defined molecular subclasses to a protein-based scoring system that could allow for a quicker and wider practicable molecular classification of GBMs in standard pathology laboratory.
settings. To this end we classified a cohort of 167 brain malignancies clinically diagnosed as primary GBMs into previously described PN, CLAS and MES subclasses. The brain tumor specimens from the classified subpopulations were subsequently studied in greater detail in several other studies that are part of this thesis.

A collection of 30 surgical specimens was used in Chapter 4 to determine the kinase activity profiles of GBM patients both in relation to the molecular subclasses and overall survival of patients. Particularly in relation to survival we identified a limited number of phosphorylation sites that could be promising subjects for future experimental studies.

The effect of two micro-environmental cues on subclass identity was then assessed in GBM models. Chapter 5 shows that TGF-β is able to induce a MES transition of GBM cells that is associated with an increase in invasive behavior of these cells. Similarly, we show in Chapter 6 that a lowered oxygen pressure can also induce a MES transition of GBM cells that is also mediated through a shared signaling pathway.

In the remainder of the thesis the focus is on angiogenesis in GBM. This second part begins with a review of the literature in Chapter 7 that outlines the importance of the Angiopoietin-2 (Ang-2) and vascular endothelial growth factor A (VEGFA)-signaling pathways in GBM angiogenesis. In addition, the role of the microenvironment GBM cells potentially reside in, is taken into account in this review.

Chapter 8 then resides at the bridge between the first and the second part of the thesis. In this chapter the vascularization patterns and levels of angiogenic factors in molecular subclasses of GBM are assessed. Morphometrical analyses pointed out that MES GBMs have larger vessels, and IL-8 is thereafter identified in Chapter 9 as a potentially important factor for vessel enlargement. Here we find that IL-8 associates with a pro-angiogenic and MES phenotype in GBMs and blocking IL-8 signaling only exerted anti-angiogenic effects through MES GSCs.

In Chapter 10 we address the interplay of the Ang-2 and VEGFA-signaling axes, and we identified an in vitro anti-angiogenic effect of exogenous dual stimulation with both factors. These anti-angiogenic effects could not be reproduced in an in vivo xenograft model. Alternatively, the effect of combined inhibition of both pathways is further assessed in Chapter 11. The inhibition of the Angiopoietin-2-signalling axis interestingly resulted in the formation of clinically diagnostic necrotic areas in GBM xenograft models, but the combination therapy with the approved drug Bevacizumab for recurrent GBM did not provide indications for synergistic activity.

The thesis is experimentally concluded with Chapter 12 that reports work on the relatively novel agent Klotho that has been assessed and applied successfully as an anti-tumorigenic agent in other tumor entities. We observed that Klotho in vitro exerted a GBM cell-mediated anti-angiogenic effect, but it did not impair in vivo angiogenesis or tumor progression in a xenograft model.

Finally, in Chapter 13 the experimental results of this thesis are summarized and we provide an integral discussion of the individual chapters. To conclude, we provide our perspective on experimental GBM research and the therapeutic potential of anti-angiogenic therapy.
References


Part I

Molecular Heterogeneity