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The unfolded protein response in glioblastoma stem cells: towards new targets for therapy

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Chapter 1

General introduction and thesis outline

General Introduction

Glioblastoma and treatment

Glioblastoma multiforme (GBM) is one of the most lethal and prevalent brain tumors among adults with a poor prognosis [1,2]. Since 2005, standard treatment of GBM is according to the Stupp protocol, surgery followed by temozolomide (TMZ) and radiotherapy [3]. Since 2016, GBM is classified by the World Health Organization (WHO) into Isocitrate dehydrogenase gene *(IDH)1* wildtype *(wt)* primary grade IV glioma, representing 90% of GBMs, and *IDH1-mutant* (mut) being mostly secondary GBM, representing 10% of diagnosed GBMs. Primary GBM arises de novo that is different from secondary GBM developing from lower grade gliomas (LGG). The mutated *IDH1* gene encodes an enzyme with disrupted catalytic activity resulting in production of the oncometabolite 2-hydroxyglutarate (2-HG). An important consequence of 2-HG is that it alters both histone and DNA methylation patterns resulting in epigenetic changes and creating a CpG island methylator phenotype (G-CIMP) in GBM [4]. The methylation status of the O6-methylguanine-DNA methyltransferase *(MGMT)* gene is associated with better prognosis and response to Temozolomide (TMZ). *IDH-mut* GBM has a more favorable prognosis than *IDH-wt* GBM, a medium overall survival of 31 months compared to 15 months after diagnosis, respectively [5].

Despite extensive current treatment of GBM the prognosis remains poor, including after the use of anti-angiogenic therapy with Bevacizumab (Avastin) as second line in relapsed tumors [6]. Several features of the tumor contribute to the lack of success of current therapy. GBM is characterized by highly infiltrative growth in the brain, which makes complete resection of the tumor impossible, decreasing the chance of a favorable outcome [7]. Furthermore, high chemo-radiotherapy resistance of GBM cells increases the occurrence of tumor relapse and a deadly outcome. Several mechanisms of resistance have been identified, for instance amplification of the EGF receptor *(EGFR)* gene present in around 50% of GBMs, results in EGFR overexpression and activation of the PI3K/AKT/mTOR survival pathway inhibiting the apoptotic response induced by chemotherapy. In addition, frequently mutated *PTEN* as well as *TP53* mutations occurring in the majority of GBMs also facilitate tumor cell growth and apoptosis resistance [8]. Efforts to develop molecular strategies to overcome for

example TMZ resistance include pre-clinical studies using RNAi to reduce expression of MGMT, p53 and EGFR proteins [9]. Furthermore, combination treatments with for example Bevacizumab and Metformin to enhance sensitivity to chemotherapy so far has not prolonged overall survival [10]. Similarly, immunotherapy with PD-1, PD-L1, and CTLA-4 checkpoint inhibitors that have demonstrated impressive efficacy in a number of tumor types have thus far not shown clinical benefit in GBM [11]. Extensive genomic characterization of GBM has identified several genes and core pathways that may be relevant for disease progression but has thus far offered no handles for improved therapy [12].

Hurdles for effective therapy: Glioma Stem Cells and cellular heterogeneity

Cancer cells resembling stem cells have been identified in various tumor types, including in GBM. These GBM stem cells (GSCs) are assumed to be the most malignant and resistant subpopulation of cells with high tumor-initiating potential and the ability to self-renew, thus driving tumor formation, progression and resistance to therapy [13-15]. Specific markers for GSCs have been reported such as cell surface receptor CD133 (Prominin) and transcription factors like SOX2, Nanog and Oct4, although providing no selective features for GSCs. Functional GSC properties such as their potent tumor forming ability in immune-incompetent mice and self-renewal potential in *in vitro* assays are considered better defining properties [16].

GSCs are considered to be main drivers for tumor recurrence and treatment failure and are therefore a major focus point for research, also in our group. Identification of novel molecular targets in GSCs will be critical for developing better therapies for this deadly disease. Patient-derived GBM spheroid models, exhibit genetic and malignant features closely resembling the original tumor and maintain cells in a more stem-like state [17,18]. To study critical pathways in GBM our lab has generated such GBM spheroid models (also named GBM neurospheres) from leftover GBM patient resected tumor material [19]. These GBM spheroids are able to differentiate and form glial, astrocyte and neuron-like offspring upon exposure to for example Fetal Calf Serum (FCS) containing medium [20].

The strong cellular heterogeneity in GBM poses another hurdle for effective

tumor eradication. Both inter- and intra-tumoral heterogeneity has been linked with failure of current treatment regimens to effectively target all GBM cell populations in the tumor [21]. Transcriptional profiling of GBM patient samples has resulted in classification into proneural (PN), classic (CL) and mesenchymal (MES) subtypes that have been linked with several principal mutations and/ or irregularities in the expression of *PDGFRA*, *IDH1*, *EGFR* and *NF1* [22]. Recently an integrated study including single cell sequencing, TCGA data analyses and in vitro experiments further demonstrated a model for classification in four cellular states, neural-progenitor-like, and oligodendrocyte-progenitor-like, astrocyte-like and mesenchymal-like associated with genetic alterations in *CDK4*, *EGFR*, *PDGFRA* and *NF1*, respectively [23]. This cellular heterogeneity can be attributed not only to genetic diversity, but also to signals derived from the tumor microenvironment (TME) and epigenetic changes that facilitate the high plasticity of GBM cells [23].

The high level of heterogeneity and plasticity of GBM cells poses obviously a major hurdle for successful GBM eradication. Novel targets need to be identified that will tackle multiple important mechanisms involved in GBM malignancy, resistance and plasticity. In this thesis we hypothesized that the ER stress/ unfolded protein response (UPR) will provide an interesting target for therapy in GBM.

ER stress/ UPR in cancer

The endoplasmic reticulum (ER) is an extensive membrane network that functions as a central intracellular organelle for protein and lipid synthesis, calcium storage and intracellular transport of proteins as well as the secretory pathway. Perturbation of ER homeostasis, such as disturbance of ATP, calcium levels or change in the redox status, can affect protein folding that leads to misfolded protein accumulation and consequently ER stress [24]. This results in the activation of an adaptive mechanism that promotes cell survival known as the UPR [25,26]. The UPR pathway is initiated via the ER transmembrane proteins IRE1, PERK and ATF6, which are kept inactive by binding to the ER chaperone BiP/GRP78. Upon stress BiP/GRP78 is released from these ER stress sensors resulting in activation of various downstream mechanisms

including the arrest of general protein synthesis, enhancement of protein folding capacity and increased RNA and protein degradation, all aiming to restore protein-homeostasis [27,28]. When ER stress is overwhelming the UPR will activate cell death programs and in cancer ER stress aggravation may provide a therapeutic strategy. In addition, autophagy is also part of the adaptive response activated by the UPR and can contribute to cell survival as well as apoptosis induction [29,30]. Autophagy is a ubiquitous catabolic process that involves the degradation of cytoplasmic components, including misfolded proteins, via the lysosomal pathway [31]. All three UPR branches have been reported to be able to activate autophagy [24,29,30].

In cancer, oncogene-driven cell proliferation has been associated with a high demand for protein production and together with conditions commonly present in tumors, like glucose/ nutrient shortage and hypoxia, result in a state of chronic ER stress and a high dependency on the UPR for cell survival [25,26]. In fact, chronic UPR activation has been linked to many of the hallmarks of cancer, including oncogenesis, proliferation, metastasis and invasion, angiogenesis, therapy resistance, signals from the TME and inflammation [32]. Furthermore, the UPR is involved in reprogramming gene expression during tumor development regulating stem cell properties in both normal and malignant stem cells [33,34]. For example, in breast cancer cells PERK was shown to be required for epithelial-mesenchymal transition (EMT), metastasis and mammospheres formation, and affected pluripotency signals [35,36]. In colon cancer stem cells UPR activation triggered differentiation, thereby influencing drug sensitivity and proliferation state [37,38]. Together this emphasizes the potential value of modulating the UPR as a therapeutic approach in cancer.

In GBM chronic activation of the UPR has been reported evidenced among others by elevated BiP/GRP78 expression [39,40]. Moreover, the UPR has been implicated in GBM growth and progression although its role in the regulation of GSCs and their highly malignant properties have remained mostly elusive [41]. Therefore, exploration of ER stress/ UPR signaling in GSCs is of great interest in order to explore its importance in regulating stemness and as a possible target for therapy in GBM.

Scope of the thesis

The main aim of the research described in this thesis was to investigate ER stress/ UPR signaling in GSCs and explore its potential as a target for therapy in GBM. The importance of the UPR in contributing to acute ER stress-induced cytotoxicity was examined, including effects on the self-renewal potential of GSCs and the underlying molecular mechanisms were elucidated. For this, previously in our lab generated and characterized patient-derived GSC-enriched GBM neurosphere models were employed.

In **chapter 2** a general introduction on the UPR and its role in cancer is provided. The current knowledge on the role of ER stress/ UPR in the development and progression of GBM and as a potential therapeutic target is evaluated.

In **chapter 3** we investigated the therapeutic potential of ER stress induction in different GBM neurosphere models. We started by analyzing the UPR activation status in GBM patients using a tissue microarray (TMA) and immunohistochemistry. Next, we examined sensitivity to the ER stress-inducing agents, thapsigargin and tunicamycin, using short-time cytotoxicity assays and the effect on self-renewal potential by long-term spheroid formation assays. The contribution of the three UPR branches in ER stress sensitivity and impact on self-renewal was determined using pharmacological inhibitors, short hairpin RNAs and CRISPR/Cas9 knockout approaches. In Chapter 3.1 (appendix), we explored correlations between expression of the hypoxic marker GLUT1 and the UPR proteins BiP/GRP78, ATF4 and XBP1 using the same clinical GBM specimens (TMA) as in chapter 3. In addition, the use of digital image analyses for determining protein levels was discussed in more detail.

In **chapter 4** we examined the effect of PERK inhibition on self-renewal potential of GSCs in the absence of acute stress and applied comparative transcriptomics to examine possible underlying mechanisms of regulation. For this, differential expression analysis on generated mRNA NGS datasets was performed obtained from controls, PERK inhibitor-treated and serum differentiated GBM neurospheres.

The involvement of autophagy in UPR-induced signaling in GBM neurospheres was addressed in **chapter 5**. In this preliminary study the activation of different autophagy molecular markers were evaluated upon extrinsic ER stress induction by thapsigargin in GBM neurospheres and differentiated counterparts and related to

thapsigargin sensitivity. The autophagic flux was studied and the transcription levels of genes involved in autophagy also were compared.

Finally, in **chapter 6** the findings of this thesis are summarized. A general discussion with conclusion of the chapters and future perspectives are presented.

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