Exploring Vulnerabilities in Malignant Glioblastoma

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To my family

ABSTRACT

Glioblastoma (GBM), the most common and malignant primary brain tumor in adults, is among the most difficult cancers to treat with a median survival of only 15 months. GBMs are highly complex tumors with several unique features explaining the lack of effective therapies: infiltrative growth of the tumor cells prevents complete surgical removal of the tumor, the blood-brain barrier (BBB) effectively inhibits drug delivery to the tumor site, and identification of subpopulations of glioma stem cells (GSCs) that are an important source of cellular heterogeneity and therapeutic resistance. Novel therapeutic approaches for treatment of these devastating tumors are urgently needed. In this study, we investigated the molecular mechanisms underlying tumor initiation, progression, and therapy resistance of malignant human GBM. We aimed at identifying vulnerabilities that could potentially provide novel therapeutic targets for treatment of GBM. We utilized patient-derived GSC cultures and patient-derived xenograft tumors as models to study GBM.

In the first study, we demonstrated that mammary-derived growth inhibitor (MDGI), also known as heart-type fatty acid binding protein 3 (H-FABP/*FABP3*), was not only highly expressed but also played a significant role in GBM invasion. We identified a novel function for MDGI in maintaining the lysosomal membrane integrity. Unexpectedly, GBM cells were extremely vulnerable to silencing of MDGI expression. We demonstrated that MDGI silencing caused lysosomal membrane permeabilization (LMP), which is an alternative cell death pathway leading to irreversible apoptosis. LMP can be induced by pharmacological agents such as antihistamines. Interestingly, we demonstrated that treatment of patient-derived xenograft tumors with antihistamine clemastine effectively eradicated the invasive tumor cells and prolonged animal survival in a preclinical study in vivo. In the most invasive patient-derived GBM model, treatment with clemastine led to a complete eradication of the tumor. Our results encourage testing clemastine in a clinical trial of patients with GBM.

In the second part of this study, we provided important insight into GSC plasticity driving tumorigenesis and therapy resistance of GBM. We identified a molecular mechanism where CD109 physically interacts with glycoprotein 130 (GP130) to regulate the interleukin-6/signal transducer and activator of transcription 3 (IL-6/STAT3) signaling pathway. We further demonstrated that the CD109/STAT3 axis was essential for the maintenance of stemness and plasticity of GSCs. When CD109 was silenced, GSCs differentiated into astrocytic-like cells and were unable to dedifferentiate into the stem-like state. Moreover, the CD109/STAT3 axis was needed for the tumorigenicity of patient-derived xenograft models in vivo. Importantly, genetic targeting of CD109 and pharmacologic inhibition of STAT3 both sensitized the GSCs to chemotherapy. These results suggest that therapeutic targeting of CD109/STAT3 axis in combination with chemotherapy might potentially increase the effect of chemotherapy in patients with GBM and help to overcome the therapy resistance. This study provides important insight into novel disease mechanisms with potential therapeutic implications for GBM patients.

TIIVISTELMÄ

Glioblastooma on aikuisten yleisin ja pahanlaatuisin aivokasvain. Nykyisillä hoitomenetelmillä glioblastoomaa ei useimmissa tapauksissa saada parannettua ja potilaiden ennuste on erittäin huono. Glioblastooman huonon ennusteen taustalla vaikuttaa monta eri tekijää. Onnistunut leikkaushoito on hankalaa, koska kasvainsolut tyypillisesti levittäytyvät eli infiltroituvat laajalle alueelle aivoissa. Lisäksi aivoissa oleva veri-aivoeste rajoittaa tehokkaasti monien lääkeaineiden kulkeutumista aivoihin. Glioblastoomissa on myös havaittu olevan kantasoluominaisuuksia omaavia kasvainsoluja. Nämä kantasolujen kaltaiset kasvainsolut ovat plastisia eli mukautuvat mikroympäristöönsä ja ovat tärkeässä osassa kasvaimen heterogeenisyyden ja lääkeresistenttiyden kehittymisessä. Uusien kohdennettujen hoitomenetelmien kehittäminen glioblastoomaan on tärkeää. Tässä tutkimuksessa selvitettiin molekyylitason mekanismeja, jotka edistävät aivokasvainten kasvua, infiltroitumista ja lääkeresistenttiyttä. Tarkoituksena oli löytää uusia haavoittuvuuksia, joita vastaan voitaisiin tulevaisuudessa kehittää kohdennettua hoitoa. Tutkimuksessa hyödynnettiin relevantteja potilaista peräisin olevia solumalleja.

Tutkimuksen ensimmäisessä osassa havaitsimme, että mammary-derived growth inhibitor (MDGI) proteiini, joka tunnetaan myös nimellä heart-type fatty acid binding protein 3 (H-FABP/*FABP3*), ilmentyy voimakkaasti erityisesti infiltroituvissa aivokasvainsoluissa. MDGI:n hiljentäminen johti yllättäen aivokasvainsolujen kuolemaan. Tutkiessamme solukuolemaan liittyviä molekyylimekanismeja osoitimme, että MDGI:llä on tärkeä rooli lysosomien kalvon rakenteen ylläpitämisessä ja sen läpäisevyyden säätelyssä. MDGI:n hiljentäminen aktivoi lysosomi-välitteisen solukuolemamekanismin aivokasvainsoluissa. Lysosomi-välitteinen solukuolema on mahdollista aktivoida myös farmakologisesti. Osoitimme, että aivokasvainsolut olivat herkkiä antihistamiini klemastiinille. Eläinkokeissa klemastiini osoittautui erittäin tehokkaaksi keinoksi päästä eroon infiltroituvista aivokasvainsoluista. Tuloksemme tukevat klemastiinin vaikutuksen tutkimista aivokasvainpotilailla kliinisessä lääketutkimuksessa.

Tutkimuksen toisessa osassa tunnistimme uuden signalointimekanismin, jonka avulla aivokasvainsoluissa ylläpidetään kantasoluominaisuuksia ja plastisuutta. Havaitsimme, että aivokasvainsolujen pinnalla ilmentyvä CD109-proteiini on vuorovaikutuksessa glykoproteiini 130:n (GP130) kanssa ja säätelee interleukiini-6/signal transducer and activator of transcription 3 (IL-6/STAT3) signalointireitin aktivoitumista. Osoitimme, että CD109:n ilmentymisen hiljentäminen lisäsi aivokasvainsolujen erilaistumista astrosyyttien kaltaisiksi soluiksi, mikä vähensi merkittävästi solujen tuumorigeenisyyttä eläinkokeissa sekä altisti solut kemoterapialle. Tulostemme perusteella CD109/STAT3 signalointireitin estämisellä aivokasvainsoluissa voisi tulevaisuudessa olla mahdollista parantaa aivokasvaimen kemosensitiivisyyttä. Tämä tutkimus on tuonut uutta ymmärrystä aivokasvaimen pahanlaatuisuuteen liittyvistä mekanismeista, jota voidaan mahdollisesti hyödyntää tulevaisuudessa tehokkaampien hoitomuotojen kehityksessä glioblastoomaan.

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ORIGINAL PUBLICATIONS

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I Le Joncour V*, **Filppu P***, Hyvönen M, Holopainen M, Turunen SP, Sihto H, Burghardt I, Joensuu H, Tynninen O, Jääskeläinen J, Weller M, Lehti K, Käkelä R, and Laakkonen P. Vulnerability of invasive glioblastoma cells to lysosomal membrane destabilization. *EMBO Mol Med.* (2019) 11(6):e9034

II **Filppu P**, Tanjore Ramanathan J, Granberg KJ, Gucciardo E, Haapasalo H, Lehti K, Nykter M, Le Joncour V, and Laakkonen P. CD109-GP130 interaction drives glioblastoma stem cell plasticity and chemoresistance through STAT3 activity. *JCI Insight* (2021) 6(9):e141486

* Equal contribution

ABBREVIATIONS

AC-like	Astrocytic-like cell state
Akt	Protein kinase B
BBB	Blood-brain barrier
CAD	Cationic amphiphilic drug
CL	Classical subtype glioblastoma
CNS	Central nervous system
CSC	Cancer stem cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
FA	Fatty acid
FABP3	Fatty acid binding protein 3
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
GBM	Glioblastoma
GEMM	Genetically engineered mouse model
GFAP	Glial fibrillary acidic protein
GP130	Glycoprotein 130
GPI	Glycosylphosphatidylinositol
GSC	Glioma stem cell
H-FABP	Heart-type fatty acid binding protein 3
HIF	Hypoxia-inducible factor
IDH1/2	Isocitrate dehydrogenase 1/2
IL-6	Interleukin 6
JAK	Janus kinase
LMP	Lysosomal membrane permeabilization
MDGI	Mammary-derived growth inhibitor
MES	Mesenchymal subtype glioblastoma
MES-like	Mesenchymal-like cell state
MGMT	O^6-methylguanine-DNA methyltransferase
MUFA	Monounsaturated fatty acid
NF1	Neurofibromin 1
NF-κB	Nuclear factor kappa B
NPC-like	Neural-progenitor-like cell state
NSC	Neural stem cell
OPC-like	Oligodendrocyte-progenitor-like cell state
PC	Phosphatidylcholine
PDGFRA/B	Platelet derived growth factor receptor alpha/beta
PDX	Patient-derived xenograft
PE	Phosphatidylethanolamine
PI3K	Phosphoinositide 3-kinase
PLA	Proximity ligation assay
PN	Proneural subtype glioblastoma
PTEN	Phosphatase and tensin homolog
PUFA	Polyunsaturated fatty acid

RB1	Retinoblastoma transcriptional corepressor 1
STAT3	Signal transducer and activator of transcription 3
TAM	Tumor-associated macrophage
TCGA	The Cancer Genome Atlas Program
TERT	Telomerase reverse transcriptase
TMZ	Temozolomide
TP53	Tumor protein 53
TTF	Tumor-treating fields
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

INTRODUCTION

Cancer is a leading cause of death, and the incidence of cancer is rising worldwide. The global cancer statistics estimated that there were 19.3 million new cancer cases and almost 10.0 million cancer deaths worldwide in 2020 (Sung et al., 2021). Among all cancers, brain and nervous system tumors were predicted to account for 1.6% of the number of new cases and 2.5% of the number of deaths (Sung et al., 2021). Although the incidence of malignant primary brain tumors is relatively low, these tumors are among the most difficult cancers to treat (Lapointe et al., 2018). Glioblastoma (GBM) is the most common malignant primary brain tumor in adults with very limited therapeutic options and dismal patient survival (Ostrom et al., 2020).

Over the past decade, GBM has been widely profiled at the molecular level (Verhaak et al., 2010; Brennan et al., 2013; Wang et al., 2017; Neftel et al., 2019). However, this increased understanding of the genomic, epigenetic, and transcriptomic landscapes of GBM has not translated into effective therapies and most targeted therapies have failed to improve patient survival (Le Rhun et al., 2019). Surgery followed by radiotherapy and temozolomide (TMZ) chemotherapy has been the standard of care already since 2005 and the survival of patients remains only about 15 months (Stupp et al., 2005; Stupp et al., 2009).

Novel effective therapies are needed to improve the extremely poor survival of GBM patients. Identification of specific vulnerabilities in tumor cells might provide means for development of more effective therapies in the future. In this study, we investigated the molecular mechanisms underlying GBM invasion and the glioma stem cell (GSC) maintenance, both associated with poor survival and therapy resistance; the invasive tumor cells evade surgical removal, and the plastic GSCs display intrinsic therapy resistance and promote the development of intratumoral heterogeneity (Gimple et al., 2019; Neftel et al., 2019).

We show that the invasive tumor cells were unexpectedly vulnerable to inhibition of their lysosomal membrane integrity. Our preclinical study demonstrated that antihistamine clemastine induced lysosome-mediated cell death and eradicated the invasive tumor cells in vivo. Our results suggest repurposing of clemastine in treatment of GBM. Furthermore, we demonstrate that CD109/STAT3 axis is essential for the maintenance of GSC plasticity, tumorigenicity, and chemoresistance suggesting that therapeutic targeting of CD109/STAT3 axis might improve the efficacy of chemotherapy in GBM patients. Our results provide insight into the molecular mechanisms underlying therapy resistance in GBM.

REVIEW OF THE LITERATURE

1. Central nervous system tumors – classification and epidemiology

Primary brain tumors comprise a heterogenous group of tumors arising in the central nervous system (CNS). The annual overall incidence rate in the United States between 2013-2017 for all primary brain and other CNS tumors was 23.79 per 100 000 people and the median age at the time of diagnosis was 60 years (Ostrom et al., 2020). About one third (29.7%) of all primary brain and other CNS tumors were malignant (Ostrom et al., 2020). Meningioma was the most common non-malignant tumor (38.3% of all tumors), whereas glioblastoma (GBM) was the most common malignant brain and other CNS tumor (14.5% of all tumors) (Ostrom et al., 2020).

Primary brain tumors are classified according to the World Health Organization (WHO) Classification of Tumors of the Central Nervous System (Louis et al., 2021). Until recently, the classification of the primary CNS tumors has largely relied on histopathology and the tumors have been graded from 1 to 4 based on their malignancy (Louis et al., 2007). In 2016, molecular parameters were incorporated in the WHO classification of the CNS tumors (Louis et al., 2016). The 2021 fifth edition has simplified the classification of adult-type diffuse gliomas and further advanced the role of molecular diagnostics in CNS tumor classification (Louis et al., 2021).

Tumors that arise from the neuroepithelial tissue of the brain are known as gliomas. Gliomas include several types of tumors for example adult- and pediatric-type diffuse gliomas, ependymal tumors, choroid plexus tumors, and pineal tumors. Embryonal tumors include medulloblastomas which occur most often in children. Other CNS tumors that do not arise from the neuroepithelial tissue but grow in the CNS include for example tumors of the cranial and paraspinal nerves, meningiomas, lymphomas, germ cell tumors, tumors of the sellar region, and metastases to the CNS (Louis et al., 2021).

1.1 Gliomas

Gliomas are one of the most common types of primary brain tumors that arise from glial cells in the CNS. According to the 2016 WHO classification of the CNS tumors, pilocytic astrocytoma is graded as grade 1 and is a non-malignant tumor that can be cured by surgical resection. Relative five-year survival rate for pilocytic astrocytoma was 94.5% (Louis et al., 2016; Ostrom et al., 2020). Grade 2-4 tumors are classified as diffuse gliomas and these tumors include WHO grade 2 and 3 astrocytic tumors, grade 2 and 3 oligodendrogliomas, and grade 4 GBMs (Louis et al., 2016).

WHO grade 2 diffuse astrocytomas and grade 3 anaplastic astrocytomas are malignant diffusely infiltrating tumors. These tumors are diagnosed at the median age of 46 and 53 years, and they have relative five-year survival estimates of 53.0% and 31.1%, respectively (Ostrom et al., 2020). Most of the grade 2 and 3 tumors have isocitrate dehydrogenase 1 or 2 (*IDH1/2*) mutation, which is associated with more favorable

prognosis (Louis et al., 2016). WHO grade 2 oligodendrogliomas and grade 3 anaplastic oligodendrogliomas are diagnosed based on the *IDH* mutation and additional codeletion of chromosomes 1p and 19q (Figure 1). Both mutations are associated with more favorable prognosis (Louis et al., 2016; Lapointe et al., 2018). The five-year survival estimates for the WHO grade 2 oligodendrogliomas and grade 3 anaplastic oligodendrogliomas were 83.4% and 64.0%, respectively (Ostrom et al., 2020). However, in the 2021 WHO classification of the CNS tumors, classification of adult-type diffuse gliomas has been simplified and these tumors are now classified only into three different tumor types: astrocytoma, *IDH*-mutant; oligodendroglioma, *IDH*-mutant and 1p/19q-codeleted; and glioblastoma, *IDH*-wildtype (Louis et al., 2021).

1.2. Other central nervous system tumors

Ependymal tumors such as ependymoma are tumors of the brain and spinal cord. Median age at the time of diagnosis was 45 years and the relative five-year survival rate for ependymomas was 90.6% (Ostrom et al., 2020). *RELA* gene fusion is typical to ependymomas, and this gene fusion is associated with poor prognosis (Lapointe et al., 2018). Meningiomas arise from the meninges that surround the brain and spinal cord. Most meningiomas are benign and grow slowly (DeAngelis, 2001). Incidence rates of meningioma increased with age and median age at diagnosis was 66 years. Meningioma was more common in females and relative five-year survival rate for meningioma was 94.2% (Ostrom et al., 2020). The most common tumor of the cranial and paraspinal nerves, also known as nerve sheath tumor, is non-malignant schwannoma (Ostrom et al., 2020). Primary CNS lymphoma accounted for 1.9% of all brain and other CNS tumors and were diagnosed at a median age of 67 years (Ostrom et al., 2020). A well-known risk factor that markedly increases the risk of primary CNS lymphoma include a compromised immune system for example due to an autoimmune disorder. Most primary CNS lymphomas are B-cell lymphomas (Buckner et al., 2007). Relative five-year survival rate for lymphomas was 37.6% (Ostrom et al., 2020).

Brain metastases are a common complication of several systemic cancers and are approximately 10 times more common than primary brain tumors. Brain metastases occur in 20-40% of patients with solid cancer and most typically originate from non-small cell lung cancer, breast cancer, and melanoma (Nguyen and DeAngelis, 2007). The incidence of brain metastases is increasing probably due to better survival of patients who have received systemic treatment for the primary tumor. The development of brain metastases has been associated with poor prognosis and treatment options are limited. Typically, treatment with surgery and radiation provides only a modest survival benefit. Relative two-year survival rate for patients with brain metastases is only about 8% (Chamberlain et al., 2017).

1.3. Glioblastoma

GBM (WHO grade 4) is the most commonly occurring primary malignant brain and other CNS tumor (48.6% of malignant tumors) (Figure 1). Annual incidence rate for

GBM was 3.2 per 100 000 people with 12011 cases reported annually in the United States. Median age at diagnosis was 65 years and incidence of GBM increased with age. The highest incidence rate 15.3 was in the age group of 75-84 years. Interestingly, GBM was also reported to be 1.59 times more common in males compared to females (Ostrom et al., 2020). GBM is the most lethal and incurable tumor with a median survival of approximately 15 months after diagnosis (Stupp et al., 2009). Relative five-year survival estimate for patients with GBM was only about 7.2% (Ostrom et al., 2020).

Based on the data from the Finnish Cancer Registry, a total of 2045 patients with GBM were diagnosed in Finland between 2000 and 2013. The incidence of GBM is increasing in Finland, especially among patients >70 years. Increased incidence of GBM can be at least partly explained by higher life expectancy and aging of the population. The median survival of all GBM patients in Finland has slightly improved from 2000-2006 to 2007-2013 from 7.5 months to 9.6 months, respectively. However, median survival times varied substantially between different age groups and survival among the elderly patients remains extremely poor (Korja et al., 2019).

The majority of grade 4 GBMs (90%) develop rapidly de novo without any evidence of lower grade tumor and are thus called primary GBMs. Secondary GBMs (10%) progress from low-grade astrocytomas (grades 2 and 3) (Ohgaki and Kleihues, 2013). Patients diagnosed with secondary GBM were significantly younger than patients with primary GBMs: mean age at the time of diagnosis was 33 and 59 years, respectively (Yan et al., 2009). Primary and secondary GBMs are histologically indistinguishable. Their characteristic pathological features include highly proliferative tumor cells, necrotic areas that are surrounded by perinecrotic pseudopalisading tumor cells, and high microvascular proliferation (Preusser et al., 2011). Primary and secondary GBMs, however, differ in their genetic and epigenetic profiles and have distinct clinical characteristics and prognosis (Ohgaki and Kleihues, 2013). *IDH1/2* mutation is a diagnostic molecular marker of secondary GBM. The mutation was observed in 85% of secondary GBMs, but only 5% of primary GBMs (Figure 1). *IDH1/2* mutation has a significant survival advantage in patients with glioblastoma. The median survival was 31 months for the GBM patients with *IDH1/2* mutation compared to 15 months for the patients with wild-type tumors (Yan et al., 2009).

In addition to *IDH1/2*-mutation, only a few prognostic biomarkers for GBM have been identified. Hypermethylation of O^6-methylguanine-DNA methyltransferase (*MGMT*) promoter has been reported in approximately 40% of *IDH* wild-type GBMs and 90% of *IDH*-mutant GBMs (Molinaro et al., 2019). Hypermethylation of the *MGMT* promoter leads to epigenetic silencing of *MGMT* gene that encodes for a DNA-repair protein and is associated with decreased DNA-repair activity after chemotherapy. In a randomized trial, *MGMT* promoter hypermethylation was an independent favorable prognostic factor and predicted benefit from temozolomide (TMZ) chemotherapy (Hegi et al., 2005). Both *IDH*-mutant astrocytoma and *IDH*-mutant GBM typically have high proportions of mutations in telomere maintenance gene *ATRX* (63% and 78%, respectively). However, *ATRX* mutation was not associated with survival advantage in these subgroups of gliomas (Molinaro et al., 2019).

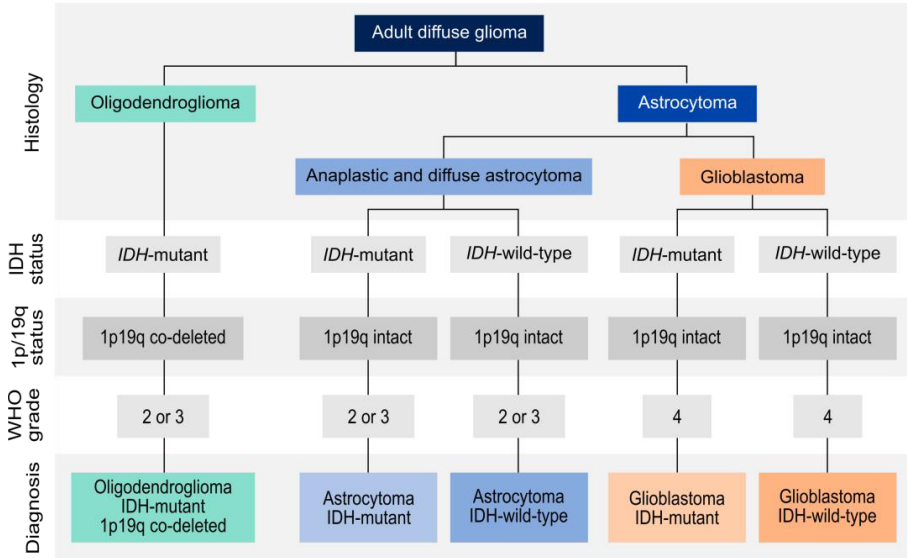

Figure 1. A simplified classification of adult diffuse gliomas based on histological and molecular features according to the 2016 WHO classification of tumors of the CNS. In addition to histological evaluation and grading, diffuse gliomas are evaluated for the IDH-mutation status. IDH-mutant gliomas are usually further tested for the 1p/19q codeletion to refine the diagnosis. The graph is modified from Louis et al., 2016; Reifenberger et al., 2017; and Molinaro et al., 2019.

The current standard of care for GBM includes maximal surgical resection of the tumor followed by radiotherapy and concomitant and adjuvant TMZ chemotherapy (Stupp et al., 2009). TMZ is an orally available and blood-brain barrier (BBB) permeable DNA-alkylating agent that induces DNA damage and cytotoxicity (Strobel et al., 2019). A randomized phase III clinical trial conducted by the European Organization for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada (NCIC) compared radiotherapy alone with radiotherapy combined with TMZ chemotherapy in patients with GBM. The study reported median overall survival of 14.6 months for patients treated with combination of radiotherapy and chemotherapy compared to 12.1 months for patients treated with radiotherapy alone (Stupp et al., 2005). Although improvement in median survival was only 2.5 months, an increase in two-year survival was approximately 16% (Stupp et al., 2009).

Tumor-treating fields (TTF) is a treatment device that delivers alternating electric fields to the scalp and causes mitotic arrest and apoptosis of rapidly dividing tumor cells (Stupp et al., 2017). TTF device has been approved by the U.S. Food and Drug Administration (FDA) as an adjuvant treatment for patients with newly diagnosed GBM (Fabian et al., 2019). The effect of TTF alone or in combination with TMZ chemotherapy was investigated in a randomized phase III clinical trial of patients with GBM who had received initial standard therapy. The study reported a significant increase in median progression-free survival of patients who received TTF plus TMZ compared to patients who received TMZ alone (6.7 versus 4.0 months, respectively). In addition, median overall survival increased from 16.0 months to 20.9 months in the

combination treatment group. No significant side effects were observed in patients who had received TTF treatment except for localized skin irritation at the treatment sites (Stupp et al., 2017).

1.3.1. Molecular heterogeneity of glioblastoma

GBM has been long observed to possess significant heterogeneity at histological level (Wen and Kesari, 2008). GBM has been extensively profiled at genomic and transcriptional levels and was the first cancer systematically characterized by the Cancer Genome Atlas Research Network (TCGA) (Cancer Genome Atlas Research, 2008). More recently, technological advancements have allowed even more comprehensive profiling of these tumors and it has become increasingly more evident that GBMs are extensively heterogeneous tumors, and the heterogeneity exhibits at multiple levels: genetic, epigenetic, and molecular (Lauko et al., 2021).

The TCGA has performed extensive profiling and molecular classification of large cohorts of GBM tumor biopsy specimens. Collectively, these studies identified some common genetic alterations among patients with GBM including tumor suppressor protein p53 (*TP53*), retinoblastoma transcriptional corepressor 1 (*RB1*), phosphatase and tensin homolog (*PTEN*), epidermal growth factor receptor (*EGFR*), neurofibromin 1 (*NF1*), telomerase reverse transcriptase (*TERT*), *IDH1*, platelet-derived growth factor receptor alpha (*PDGFRA*), and phosphoinositide 3-kinase (*PI3K*) important in driving GBM tumorigenesis (Cancer Genome Atlas Research, 2008; Verhaak et al., 2010; Brennan et al., 2013). These frequent genetic alterations affect several core signaling pathways that are critical for GBM. Majority (66%) of the tumors had genetic alterations affecting at least one receptor tyrosine kinase (EGFR, PDGFRA, MET, or fibroblast growth factor receptor (FGFR)) and 25% of the tumors had alterations in the PI3K. Collectively these alterations affect the mitogen-activated protein kinase (MAPK) and PI3K/protein kinase B (Akt) signaling pathways. Furthermore, 85% of the tumors showed deregulated p53 signaling pathway to resist apoptosis and 79% of the tumors had alterations affecting retinoblastoma signaling leading to deregulated cell cycle control via cyclin-dependent kinases (Figure 2) (Brennan et al., 2013). The best-known epigenetic alteration in GBM with prognostic value is the above-discussed *MGMT* promoter methylation (Hegi et al., 2005).

A following study classified *IDH* wild-type GBMs into three transcriptional subtypes based on their bulk RNA-sequencing profiles: mesenchymal (MES), proneural (PN), and classical (CL) (Wang et al., 2017). Moreover, each subtype is associated with specific genetic alterations: The CL subtype is characterized by high-level *EGFR* amplification and lack of *TP53* mutations whereas *NF1* deletions are typical to the MES subtype. Two major genetic features of the PN subtype include alterations in *PDGFRA* and *IDH1* point mutations (Verhaak et al., 2010). PN subtype typically occurs in younger patients and has a survival advantage likely due to the high frequency of *IDH* mutation. MES subtype is associated with the worst survival and higher percentage of necrosis and inflammation-associated genes, as well as increased infiltration of tumor-associated glial cells and microglia/macrophages. CL subtype

tends to be more responsive to intensive therapy (Verhaak et al., 2010; Wang et al., 2017).

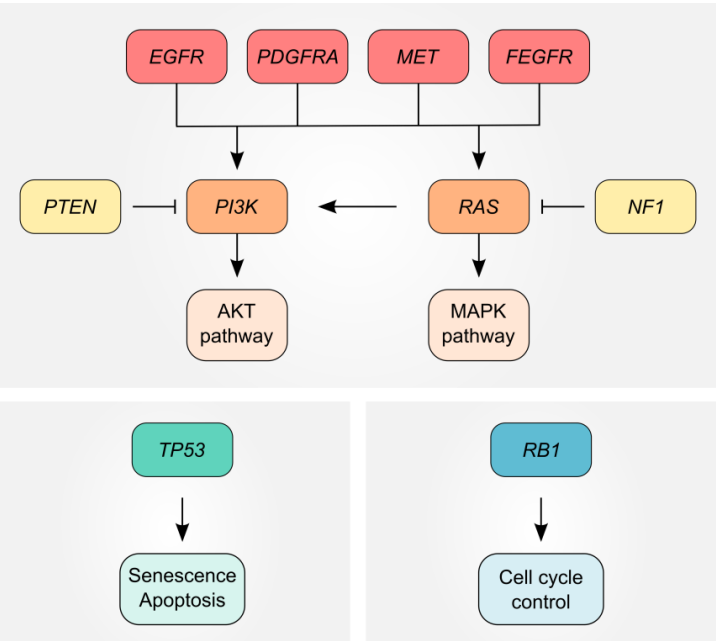

Figure 2. The most central genetic alterations in GBM and associated signaling pathways. Mutations in receptor tyrosine kinases (*EGFR, PDGFRA*, *MET*, and *FGFR*) and tumor suppressor genes *TP53* and *RB1* are frequent in GBM and lead to dysregulated signaling pathways that promotes cell proliferation and inhibits apoptosis. Modified from Brennan et al., 2013.

Sottoriva et al. were the first to demonstrate intratumoral heterogeneity in GBM. They developed a surgical multisampling approach and collected several spatially distinct tumor fragments from GBM patients. By comparing gene expression levels, they reported that different GBM subtypes were present within a single tumor as evidence of extensive intratumoral heterogeneity (Sottoriva et al., 2013). Advancements in the single-cell RNA-sequencing technology have allowed transcriptional profiling of single cells which provides a powerful tool to study intratumoral heterogeneity (Patel et al., 2014; Neftel et al., 2019). Recent single-cell RNA-sequencing study showed that GBM cells exist in four main cell states: mesenchymal-like (MES), oligodendrocyte-progenitor-like (OPC-like), neural-progenitor-like (NPC-like), and astrocyte-like (AC-like). Moreover, these cell states are dynamic and tumor cells have a capacity to transition between different cell states (Neftel et al., 2019). Cell state transition is also known as cellular plasticity and this topic will be discussed in more detail later. Extensive intratumoral heterogeneity is considered as one of the key factors underlying therapeutic resistance of GBM.

2. The microenvironmental landscape of glioblastoma

Tumors are seen as complex ecosystems where the tumor microenvironment is an integral component and plays a critical role in promoting tumor progression and therapy resistance (Figure 3) (Prager et al., 2019). There are several unique characteristic features that make GBMs as one of the most difficult types of tumors to treat with currently no effective therapies. GBMs are characterized by diffuse infiltration of tumor cells into the normal brain parenchyma, which prevents complete surgical removal of the tumor (Cuddapah et al., 2014). GBMs are highly hypoxic tumors with prominent necrotic regions that are surrounded by pseudopalisading areas and hypoxic tumor cells. GBMs are also among the most angiogenic tumors with high microvascular proliferation (Hambardzumyan and Bergers, 2015). Furthermore, presence of the BBB effectively inhibits the delivery of chemotherapeutic drugs into the brain (Cuddapah et al., 2014). The brain as an organ also provides many highly unique features that are important for tumor growth: the presence of the BBB, distinctive composition of the extracellular matrix, unique brain-resident cells including microglia, neurons, and astrocytes, and generally high degree of immunosuppression (Quail and Joyce, 2017). Another important aspect is the modulation of the tumor microenvironment by reciprocal communication between tumor cells and normal brain cells through secreted cytokines, chemokines, or direct cell-cell interactions (Broekman et al., 2018).

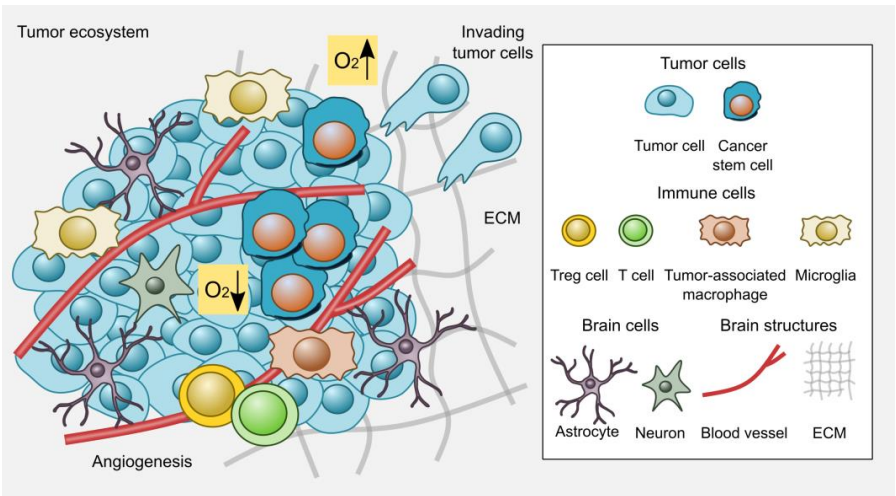

Figure 3. GBM tumor microenvironment. In addition to the tumor cells, the tumor microenvironment consists of immune cells, brain-resident cells, blood vessels, and extracellular matrix (ECM). GBM cells are highly invasive, and the tumors are characterized by hypoxic regions and high degree of angiogenesis. The graph is modified from Quail and Joyce, 2017 and Broekman et al., 2018.

2.1. Brain tumor vasculature and the blood-brain barrier

The brain is a very highly vascularized organ. Blood vessels maintain normal tissue homeostasis by providing oxygen and nutrient supply to tissues and clearing waste products (Nagy et al., 2010). Similarly, tumor cells require blood vessels to support tumor growth and progression. Brain vasculature is structurally and functionally highly unique. Endothelial cells form the walls of the blood vessels and are connected by tight junctions. Pericytes cover the abluminal surface of the vessels. Endothelial cells and pericytes are surrounded by basement membrane that is composed of extracellular matrix proteins such as type IV collagen, laminins, fibronectin, and proteoglycans (Baluk et al., 2005; Daneman and Prat, 2015). Moreover, astrocytic endfeet cover almost the entire cerebrovascular surface (Watkins et al., 2014). Endothelial cells, pericytes, and their basement membrane together with astrocytes comprise the BBB.

The BBB is a physiological barrier and critical regulator of CNS homeostasis (Daneman and Prat, 2015). It provides unique protection of CNS tissue for example from pathogens and inflammation and specifically controls the uptake of molecules. The BBB has low passive permeability to cells, pathogens, toxic substances, and many other macromolecules. However, endothelial cells express specific transporters for uptake of essential solutes and nutrients and ion channels to maintain optimal ionic composition (Abbott et al., 2010). At the same time the BBB represents a major therapeutic challenge for treatment of GBM since it effectively limits the delivery of chemotherapeutics into the brain (Quail and Joyce, 2017).

Tumor blood vessels are structurally highly aberrant, hierarchically disorganized, and functionally abnormal (Nagy et al., 2010). Tumor blood vessels vary in size and shape and are irregularly branched. Moreover, tumor blood vessels typically lack the tight endothelial junctions that together with defective pericyte coverage result in increased permeability, leakiness, and hemorrhages (Baluk et al., 2005). Tumor growth is highly dependent on adequate blood supply. Angiogenesis, sprouting of new vessels from the pre-existing ones, is essential for normal physiological processes such as embryogenesis and wound healing, but also an important mechanism for tumors to acquire their vasculature (Figure 3) (Baluk et al., 2005). In many tumors, angiogenesis is the primary source of vessel formation and mechanism to acquire vascular supply (Donnem et al., 2018).

Tumor growth pressure combined with highly glycolytic tumor cells and dysfunctional blood vessels generates a microenvironment that is acidic and hypoxic which consequently leads to formation of necrotic regions (Figure 3) (Nagy et al., 2010). Hypoxia stabilizes the transcription factor hypoxia inducible factor 1 (HIF1), which induces angiogenesis by upregulating vascular endothelial growth factor (VEGF) expression. VEGF is required for the maintenance of endothelial cells and is a central stimulator of angiogenesis and signals via its receptors expressed on endothelial cells (Nagy et al., 2010). VEGF is upregulated in GBM and expressed by multiple cell types including glioma cells to induce angiogenesis. VEGF also increases vascular permeability, which leads to increased interstitial pressure and edema and decreased delivery of chemotherapeutic drugs (Tate and Aghi, 2009). In highly vascularized tissues such as brain, an alternative way for tumor cells to get access to

blood vessels instead of angiogenesis is vessel co-option. In a process of vessel co-option, tumor cells migrate towards and along the pre-existing blood vessels. Importantly, vessel co-option is also a strategy for diffusively infiltrating glioma cells to invade into the normal brain parenchyma (Seano and Jain, 2020).

Alternative neovascularization mechanisms in tumors are vascular mimicry and transdifferentiation of tumor cells into endothelial cells (Soda et al., 2013). In vascular mimicry, tumor cells establish vascular-like networks. In GBM, vascular mimicry has been demonstrated in both mouse models (Shaifer et al., 2010) and in patients (El Hallani et al., 2010) and mechanistically might involve VEGFR2 (Soda et al., 2013). Transdifferentiation of GBM cells into endothelial cells in human GBM have been reported in a few studies (Ricci-Vitiani et al., 2010; Wang et al., 2010), but this requires further investigation. It has been suggested that both alternative neovascularization mechanisms are mediated by GSCs and may contribute to anti-angiogenic therapy resistance in GBM (Soda et al., 2013).

2.2. Invasion

Diffusively infiltrative growth is one of the main causes of poor therapeutic outcome of patients with gliomas (Figure 3). In contrast to other advanced-stage tumors that rely on blood vessels and lymphatics for systemic dissemination and metastasis formation, gliomas rarely metastasize outside the brain. This might be possibly due to inability of glioma cells to enter the vasculature or survive outside the CNS tissue. Another limiting factor might be the very short overall survival of glioma patients as metastasis formation as the final stage of cancer takes time (Cuddapah et al., 2014).

The brain extracellular matrix (ECM) is highly unique and differs from other organs (Figure 3). The brain ECM can be divided into two groups: the parenchymal ECM and the basal membranes that surround blood vessels. The parenchymal ECM consists mainly of glycosaminoglycans such as hyaluronic acid and proteoglycans that form a three-dimensional (3D) network. The basal membranes are rich in collagens, especially collagen IV, glycoproteins such as laminin, and other adhesion molecules (Ferrer et al., 2018). Mechanical rigidity of the ECM regulates GBM cell invasion. For example, GBM cells invaded extensively in more rigid ECM compared to less rigid ECM (Ulrich et al., 2009).

Glioma cells have two preferential pathways for invasion: perivascular space and white matter tracts of the brain parenchyma (Cuddapah et al., 2014). Glioma cells infiltrate the brain in different growth patterns: single cell infiltration, co-optive infiltration, and collective infiltration. Gliomas can also grow as a non-infiltrating pattern with a discrete border of tumor-brain parenchyma. Moreover, tumor growth requires access to the blood supply. Glioma cells can utilize either angiogenesis or vessel co-option, which is a non-angiogenic mechanism to access the vasculature, or sometimes even a combination of both mechanisms (Donnem et al., 2018).

Single cell infiltration of glioma cells resembles the migration of normal neural stem cells (NSCs) that often exploit the pre-existing structures of the brain such as nerve tracts of the white matter or blood vessels (Cuddapah et al., 2014). In co-optive or

perivascular infiltration glioma cells bind to the abluminal surface of existing blood vessels and migrate along it (Kuczynski et al., 2019). In general, single cell infiltration and vessel co-option, the non-angiogenic glioma growth patterns, preserve the existing tissue structures in the brain including the BBB (Donnem et al., 2018; Griveau et al., 2018). Co-optive tumor cells can, however, cause limited alterations to the properties of the BBB by displacing pericytes and astrocytes covering the vessels during migration (Watkins et al., 2014; Kuczynski et al., 2019).

Collective infiltration and non-infiltrating growth usually require activation of angiogenesis, which damages the tissue structures in the microenvironment including the BBB. The BBB breakdown has severe consequences to the tissue such as increase of vascular permeability, induction of chronic inflammation in the tumor stroma, activation of inflammation-related signaling pathways and resident microglia, and recruitment of immune cells (Abbott et al., 2010; Donnem et al., 2018; Griveau et al., 2018). Invasion mechanism and interactions of glioma cells with the vasculature are plastic. Glioma cells employ different strategies depending on the properties of their microenvironment, stage of the disease, and/or in response to treatment (Griveau et al., 2018; Kuczynski et al., 2019).

2.3. Immunosuppression

GBMs are among the most immunosuppressive tumors and have several mechanisms to suppress immune responses (Grabowski et al., 2021). GBM cells themselves upregulate and secrete several immunosuppressive cell surface factors and cytokines including for example transforming growth factor beta (TGF-β), IL-1, IL-10, and fibrinogen like 2 (FGL2), all of which suppress effector activities of immune cells (Nduom et al., 2015). Moreover, GBM cells promote local immune dysfunction by attracting tumor-associated macrophages (TAMs) to the tumor microenvironment and promoting their polarization toward the anti-inflammatory M2 phenotype to suppress adaptive immune responses (Figure 3). Both described immunosuppressive mechanisms support tumor progression (Medikonda et al., 2021).

In addition to the immunosuppressive effects, GBM cells also promote various modes of T cell dysfunction. Previous studies have demonstrated that particularly a subset of immunosuppressive regulatory T cells (Tregs) is expanded in patients with gliomas (Figure 3). A characteristic feature of Tregs is their ability to suppress effector T cell activation and function (Grabowski et al., 2021). Fecci and colleagues detected an increased fraction of Tregs in the blood samples of patients with gliomas (Fecci et al., 2006). Another study reported a prominent infiltration of immunosuppressive Tregs within glioma tumor tissue suggesting that Tregs may contribute to the lack of effective immune activation against gliomas (Hussain et al., 2006).

Among the various mechanisms of T cell dysfunction in GBM, T cell exhaustion is particularly severe. T cell exhaustion results from a prolonged antigen exposure and as a consequence, increased expression of several coinhibitory receptors such programmed cell-death protein 1 (PD-1) and cytotoxic T-lymphocyte associated protein (CTLA4) on the surface of T cells leading to their dysfunction (Woroniecka et al., 2018).

2.4. Crosstalk with normal brain cells

2.4.1. Tumor-associated macrophages

Tumor-associated macrophages (TAMs) comprise the majority of immune cells within the brain, even up to 30% of the tumor mass (Graeber et al., 2002). GBM microenvironment contains TAMs at least from two different sources: brain-resident microglia and bone marrow-derived infiltrating monocytes that differentiate into macrophages (Figure 3) (Broekman et al., 2018). TAMs have been traditionally classified based on their activation states into pro-inflammatory and tumor suppressive (M1) or tumor promoting (M2) phenotypes. The traditional model of TAM activation was based on in vitro studies and has now been questioned. Accumulating evidence demonstrates that TAM polarization is far more complex and context-dependent than previously thought (Ransohoff, 2016; Broekman et al., 2018).

Glioma cells can release several factors, including monocyte chemoattractant protein 1 (MCP-1) and MCP-3, colony-stimulating factor 1 (CSF-1), and granulocyte-macrophage colony stimulatory factor (GM-CSF), stromal cell-derived factor 1 (SDF-1), and hepatocyte growth factor (HGF), to attract TAMs to infiltrate the tumor. On the other hand, TAMs can release a wide range of growth factors and cytokines in response to signals from tumor cells and also have immune functions (Hambardzumyan et al., 2016). TAMs support tumor progression by induction of angiogenesis, tumor cell proliferation and invasion. TAMs secrete several cytokines and pro-angiogenic chemokines such as VEGF and CXC-chemokine ligand 2 to promote angiogenesis. Alternatively, CSF-1 secreted by GBM cells induces insulin-like growth factor-binding protein 1 (IGFBP1) release by TAMs which also promotes angiogenesis. GBM cells secrete inactive pro-MMP2 whereas microglia in the tumor microenvironment release MT1-MMP, which then cleaves the pro-MMP2 into active form and promotes GBM cell invasion (Hambardzumyan et al., 2016; Broekman et al., 2018).

2.4.2. Astrocytes

Astrocytes are glial cells and represent one of the most abundant cell populations in the CNS (Figure 3) (Sofroniew, 2020). Astrocytes are essential for supporting several normal physiological functions in the healthy CNS including for example formation of synapses, providing metabolic support to neurons, contribution to CNS metabolism, modulation of blood flow, and formation and maintenance of the BBB (Sofroniew and Vinters, 2010). An intriguing finding that brain tumor cells can form specialized membrane protrusions called microtubes and utilize them as routes for invasion and formation of malignant multicellular networks was groundbreaking in the field (Osswald et al., 2015). Specifically, Osswald and colleagues demonstrated that glioma cells form specialized and functional gap junctions with astrocytes composed of connexin 43 within the brain TME to mediate their resistance to radiotherapy (Osswald et al., 2015). Similar specialized gap junctions between tumor cells and astrocytes were later demonstrated in brain metastases. Paracrine signaling between astrocytes and brain metastatic cells activated STAT1 and nuclear factor kappa B (NF-

κB) signaling in tumor cells, which promoted tumor growth and chemoresistance (Chen et al., 2016).

In response to CNS injury or disease, astrocytes undergo a profound phenotypic transformation known as reactive astrocytosis where they become activated (Liddelow et al., 2017). More specifically, two different types of reactive astrocytes have been identified, A1 and A2, induced by neuroinflammation and ischemia, respectively. The A1 reactive astrocytes were shown to be harmful to neural cells whereas the A2 reactive astrocytes associated with neuroprotective functions (Liddelow et al., 2017). The functions of reactive astrocytes have only recently begun to emerge and therefore the current understanding about the roles of reactive astrocytes in cancer development and tumor progression is very limited. Recently, reactive astrocytes were demonstrated to promote formation of brain metastases via a mechanism involving activation of STAT3 (Priego et al., 2018). In GBM, reactive astrocytes were shown to promote formation of immunosuppressive TME (Henrik Heiland et al., 2019).

2.4.3. Neurons

Interactions between glioma cells and normal neural cells (Figure 3) have been relatively unexplored until recent groundbreaking discoveries that have emerged in the field. A recent study reported that neuronal activity promotes glioma growth via paracrine signaling mechanisms involving the synaptic adhesion molecule neuroligin-3 (Venkatesh et al., 2015). In the follow-up study, exposure of glioma cells to neuroligin-3 induced expression of several synaptic genes in glioma cells (Venkatesh et al., 2017). Furthermore, two independent studies demonstrated for the first time that gliomas formed neuron-glioma synapses, which promoted tumor progression (Venkataramani et al., 2019; Venkatesh et al., 2019). Genetic or pharmacologic inhibition of neuron-glioma synapses impaired proliferation and invasion of glioma cells. To conclude, these two studies provide the first evidence of direct synaptic glioma-neuron communication which holds potential for clinical implications (Venkataramani et al., 2019; Venkatesh et al., 2019).

3. Glioblastoma stem cells

3.1. Identification and enrichment of glioblastoma stem cells

The first scientific evidence of existence of cancer stem cells (CSCs) was published in 1997, when Dick and colleagues described the isolation of a specific cell population capable of initiating acute myeloid leukemia (AML) in mice and named them as leukemia-initiating cells (Bonnet and Dick, 1997). They reported that these tumorigenic CSCs also possessed proliferative and differentiative capacities and were able to self-renew, thus forming a basis for functional characterization of CSCs (Bonnet and Dick, 1997). Soon after, CSCs were reported in many solid tumors including breast (Al-Hajj et al., 2003), prostate (Collins et al., 2005), colorectal (O'Brien et al., 2007; Ricci-Vitiani et al., 2007), and pancreatic (Li et al., 2007) cancers as well as brain tumors (Hemmati et al., 2003; Singh et al., 2003).

CSCs in GBM have been accordingly named as glioma or glioblastoma stem cells (GSCs). Initial work in GBM identified CD133 as a marker to enrich GSCs and demonstrated that CD133+ GSCs enriched by flow cytometry have the ability to self-renew and initiate tumors when transplanted into the animal brain. The authors also demonstrated that injection of as few as 100 CD133+ GSCs gave rise to tumors whereas injection of 10^5 CD133- non-GSCs failed to initiate tumors in xenograft studies (Singh et al., 2004). Subsequent studies, however, demonstrated that also CD133- tumor cell population isolated from primary GBM showed stem cell-like characteristics in vitro, such as sphere-forming ability, were able to differentiate into different neural lineage cells, and were tumorigenic when transplanted into the animal brain suggesting that brain tumors contain multiple different populations of GSCs (Beier et al., 2007; Joo et al., 2008). Moreover, another study by Wang and colleagues demonstrated that CD133- GSCs were tumorigenic when transplanted into the rat brain and further analysis showed that the formed tumors contained CD133+ tumor cells suggesting that CD133+ population was generated during the tumor progression (Wang et al., 2008).

Since CD133 is also expressed by hematopoietic stem cells, NSCs, and endothelial progenitor cells, several studies have questioned its relevance as a marker for GSCs (Brescia et al., 2012). Moreover, it has been proposed that only the glycosylated cell surface protein rather than mRNA expression of CD133 gene, *PROM1*, would be a marker for GSCs. Despite the controversies, CD133 remains the most used cell surface marker for GSC enrichment (Lathia et al., 2015). Other markers used for GSC enrichment include both cell surface molecules and intracellular transcription factors such as NESTIN (Tunici et al., 2004), CD15 (Son et al., 2009), SOX2, Olig2, NANOG (Ligon et al., 2007; Suva et al., 2014), L1 cell adhesion molecule (L1CAM) (Bao et al., 2008), CD44 (Liu et al., 2006), and integrin α6 (Lathia et al., 2010). Since a single marker or a combination of markers that would specifically enrich GSCs do not exist, functional assays demonstrating the GSC properties such as self-renewal, differentiation potential, and tumorigenicity are essential in characterizing GSCs.

3.2. Functional characterization and clinical relevance of glioblastoma stem cells

It has been generally accepted in the field that expression of CSC markers is not enough to characterize the existence of CSCs and their functional validation is essential to assess stem cell characteristics of isolated cell populations. The functional validation includes assays testing cell proliferation and self-renewal, differentiation potential, tumorigenicity in vivo (Lathia et al., 2015). To summarize, functionally defined GSCs should be able to self-renew, differentiate into multiple different cell types, be tumorigenic upon orthotopic injection, and capable of recapitulating the cellular heterogeneity present in the original tumor (Lee et al., 2006).

GSCs have been reported to play important roles in mediating tumor progression and therapy resistance in GBM. Hypoxia has been shown to regulate survival and self-renewal of both NSCs and GSCs, but hypoxia also promotes tumorigenicity of GSCs via a mechanism involving HIF-2α (Clarke and van der Kooy, 2009; Heddleston et

al., 2009; Li et al., 2009). Hypoxia has also been shown to upregulate stemness markers as an indication of increased self-renewal of GSCs (Heddleston et al., 2009). GSCs have been shown to promote angiogenesis by secreting VEGF and grow in a highly infiltrative pattern in xenograft models of GBM (Wakimoto et al., 2009).

Another clinically relevant feature of GSCs is their therapeutic resistance. GSCs have intrinsic therapy resistance mainly due to their quiescence, but also mechanisms to resist radiation and chemotherapy (Saygin et al., 2019). It has been previously demonstrated that GSCs activate the DNA damage checkpoint kinases in response to radiation and have increased capacity to repair DNA damages (Bao et al., 2006). Another study demonstrated that CD133+ GSCs were significantly more resistant to several chemotherapeutic agents including TMZ compared to CD133- non-GSCs (Liu et al., 2006). Furthermore, a study using a genetically engineered mouse model of glioma demonstrated that GSCs repopulate brain tumors after therapy suggesting that GSCs promote tumor recurrence (Chen et al., 2012).

3.3. Glioblastoma stem cells reside in specific niches

GSCs reside in specific niches throughout the tumor: the leading edge, the perivascular niche, and the hypoxic-necrotic core region (Prager et al., 2020). In the stem cell niche GSCs interact with different tissue resident cells via secreted factors or direct cell-cell contacts which maintains their stemness. GSCs in the leading edge represents a clinical challenge because these cells cannot be surgically resected and therefore are a potential source for tumor recurrence. GSCs in the leading edge have been demonstrated to upregulate L1CAM and ephrin B2 signaling pathways to promote invasion (Prager et al., 2020). Moreover, GSCs have been shown to express several mediators of epithelial-mesenchymal transition (EMT), such as twist family BHLH transcription factor 1 (TWIST1), STAT3, and NF-κB, to acquire a migratory phenotype required for invasion (Prager et al., 2020).

In the perivascular niche, GSCs are in close contact with tumor-associated vasculature. Endothelial cells promote the maintenance of GSC stemness phenotype through activation of various signaling pathways such as NOTCH, sonic hedgehog, and nitric oxide (NO). Endothelial cells have been shown to release NO, which activates the NOTCH signaling in GSCs and promotes their stemness and tumorigenicity in vivo (Charles et al., 2010). Interactions within the perivascular niche are not unidirectional, since GSCs have been demonstrated to secrete VEGF to promote angiogenesis in xenograft models of GBM (Bao et al., 2006). Some interesting yet controversial evidence suggests that GSCs could give rise to endothelial-like cells (Ricci-Vitiani et al., 2010; Wang et al., 2010) or even transdifferentiate into vascular pericytes to support tumor growth (Cheng et al., 2013).

The hypoxic and necrotic regions are a hallmark of GBM and associated with increased stemness and resistance to therapies (Heddleston et al., 2011). The hypoxic response in cells is mostly mediated by the HIF1 and HIF2. HIF1is a key mediator of acute hypoxia and activates pro-survival effects in tumor cells through the PI3K/Akt and the extracellular signaling related kinase (ERK) 1/2 signaling pathways. HIF1 also upregulates the expression of VEGF in endothelial cells to promote angiogenesis. In

contrast, HIF2 levels are elevated during chronic hypoxia, which has been shown to upregulate expression of stemness factors including SOX2, OCT-4, and NANOG. Importantly, hypoxia has been suggested to promote a quiescent GSC phenotype that could significantly enrich chemo- and radioresistant GSC populations (Heddleston et al., 2011; Prager et al., 2020). To summarize, the tumor stem cell niches are dynamic and provide specific microenvironmental cues and cell-cell communication to support GSCs maintenance and to promote therapy resistance.

3.4. Models of glioblastoma heterogeneity

GBMs are characterized by extensive cellular heterogeneity. Phenotypic and functional heterogeneity among cancer cells can arise from different sources including genetic changes, microenvironmental differences, and reversible changes in cell properties also known as cellular plasticity (Meacham and Morrison, 2013). Previous studies suggest that intratumoral heterogeneity is an important contributor to treatment failure and therapeutic resistance in GBM (Sottoriva et al., 2013; Piccirillo et al., 2015).

Two models have been predominating in explaining the high cellular heterogeneity that is observed in GBM: the clonal evolution model and the cancer stem cell (CSC) model (Figure 4). According to the clonal evolution model, also known as the stochastic model, genetic and epigenetic mutations accumulate over time, which give a selective growth advantage to certain cells. As a result, any cell may have tumorigenic potential in theory and the selective pressure drives the tumor heterogeneity. The CSC model, also known as the hierarchical model, proposes that a small subpopulation of cells with stem-like properties drive tumor initiation and cancer progression. The CSC model also proposes that tumors are hierarchically organized like normal tissues and that the CSCs give rise to a variety of cell types including intermediate progenitor cells (transit amplifying cells) and more differentiated progeny, and therefore, the CSCs are the source of cellular heterogeneity in tumors. The clonal evolution model and the CSC model are not mutually exclusive (Rich, 2016).

An alternative model based on cellular plasticity has been proposed to link the clonal evolution and the CSC models. The plasticity model proposes that differentiated cancer cells can de-differentiate to cancer stem-like cells by dynamic cell state transitions, thus adding one more layer of complexity (Figure 4) (Rich, 2016). Two recently published studies provide strong evidence that supports the plasticity model in explaining GBM cell heterogeneity. Neftel et al. demonstrated by single-cell RNA-sequencing that GBM cells exist in four main cell states and exhibit a high level of cellular plasticity between the different states (Neftel et al., 2019). Dirkse et al. showed that the phenotypic heterogeneity observed in GBM results from reversible cell state transitions that are driven by the tumor microenvironment (Dirkse et al., 2019). GBM cell plasticity will be discussed in more detail in the following section.

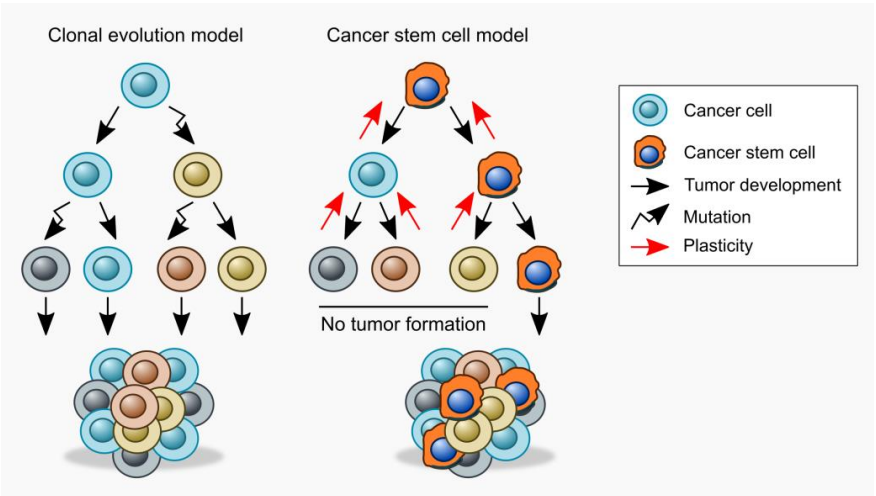

Figure 4. Clonal evolution and cancer stem cell models in tumor development and progression. According to the clonal evolution model, mutations accumulate over time and any cell in each cell population may have tumorigenic potential. In the CSC model, only tumorigenic CSCs can initiate tumor growth. The clonal evolution and CSC models are not mutually exclusive. According to the Plasticity model, differentiated tumor cells can dedifferentiate to a CSC state and acquire tumorigenic properties. The graph is modified from Rich, 2016.

3.5. Plasticity in glioblastoma stem cell networks

A cell state transition or alternatively a phenotypic switch in cell fate is also known as cellular plasticity. Cellular plasticity is typical to stem cells and highly essential during normal physiological processes like tissue regeneration and wound healing. However, also more differentiated cells can retain potential to switch their cell fate for example through reacquisition of stemness properties or transdifferentiating into other cell types (Gupta et al., 2019). An example of reacquisition of stem cell state was the groundbreaking discovery that a combination of only four transcription factors (Oct3/4, Sox2, Klf4, and c-Myc) could reprogram differentiated adult mouse or human fibroblasts to an embryonic stem cell-like state (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Another example of cellular plasticity is the EMT, which is associated with normal developmental processes like embryogenesis, tissue regeneration as well as cancer progression and metastasis (Kalluri and Weinberg, 2009). Activation of EMT in cancer has been linked to acquisition of stem-like properties such as increased self-renewal and expression of stem cell markers (Mani et al., 2008).

Cellular plasticity has been increasingly recognized as a source of cellular heterogeneity and therapy resistance in cancer. Cancer stem-like cells have the highest potential for adaptation due to their high degree of cellular plasticity. CSCs can respond to various stimuli such as cell-cell interactions, tumor microenvironmental cues, or therapy by dynamic cell state transitions likely explaining their therapeutic

resistance. In contrast, differentiated cells have more restricted transcriptional programs and therefore less potential for adaptation (Prager et al., 2020).

Several studies have demonstrated cellular plasticity among GSCs and its importance in tumorigenicity and therapy resistance. Expression of bone morphogenetic proteins (BMPs), particularly BMP4, in GSCs have been shown to inhibit tumorigenic potential of GSCs by inducing cell differentiation (Piccirillo et al., 2006). More recently, overexpression of a defined set of neurodevelopmental transcription factors (POU3F, SOX2, SALL2, and OLIG2) reprogrammed non-tumorigenic differentiated glioma cells into tumorigenic stem-like state (Suva et al., 2014). Furthermore, treatment of differentiated tumor cells with TMZ chemotherapy induced a phenotypic shift of the non-GSCs population to a GSC-like state (Auffinger et al., 2014).

Single-cell RNA-sequencing provides a powerful strategy to characterize cellular heterogeneity, model tumor evolution, and to study interactions with the tumor microenvironment. Indeed, recent single-cell studies have provided significant amounts of data and increased our understanding about this complex disease and its underlying heterogeneity and plasticity. First single-cell RNA-sequencing studies focused on *IDH*-mutant gliomas (oligodendrogliomas and astrocytomas) and identified cellular hierarchy with three main subpopulations: proliferative stem-like cells resembling NPC-like cells and two subpopulations of more differentiated cells resembling (AC-like) and oligodendrocytes (OC-like) cells (Tirosh et al., 2016; Venteicher et al., 2017). Thus, these results support that *IDH*-mutant gliomas would be driven by NPC-like cells that differentiate into OC-like and AC-like cells. These studies therefore are consistent with the CSC model (the hierarchical model) of tumorigenesis, described above, where the small subpopulation of stem-like cells drive tumor propagation and heterogeneity (Suva and Tirosh, 2020).

In contrast to *IDH*-mutant gliomas, in *IDH*-wild type GBMs such hierarchical organization was less clear. The first single-cell RNA-sequencing study in *IDH*-wild type GBM confirmed the previous finding and demonstrated that multiple different cell states that correspond to different GBM subtypes are present within a single tumor (Patel et al., 2014). A more recent single-cell RNA-sequencing study demonstrated that GBM cells exist in four primary cell states: MES-like, OPC-like, NPC-like, and AC-like and observed a high degree of cellular plasticity between the different cell states (Neftel et al., 2019). Moreover, upon xenotransplantation of tumor cells of a given cell state, the diversity of cell states present in the original tumors was re-established in the xenografts as the tumors were formed (Neftel et al., 2019).

An active role of tumor microenvironment has been increasingly noted as a source of cellular plasticity promoting intratumoral heterogeneity (Dirkse et al., 2019; Neftel et al., 2019). A recent study demonstrated that GSCs exist in cell states and such cell states were not hierarchically organized. Moreover, phenotypic heterogeneity in GSCs resulted from the reversible state transitions that were guided by the tumor microenvironment (Dirkse et al., 2019). Taken together, the CSC model in GBM has extensive experimental evidence. However, recent studies have challenged the hierarchical CSC model in GBM, and growing evidence supports the plasticity model (Dirkse et al., 2019; Neftel et al., 2019; Suva and Tirosh, 2020). This remains an active

area of investigation and the field is currently shifting towards identifying therapies that would target the GBM cellular plasticity (Prager et al., 2020).

4. Therapeutic strategies in glioblastoma

4.1. Emerging therapies

Increased understanding and recent groundbreaking discoveries in GBM biology have not translated into effective therapeutics. The standard of care for GBM still mostly relies on surgery with radiotherapy and TMZ chemotherapy, and patient survival remains very poor (Stupp et al., 2009). Despite significant efforts, no therapeutic intervention that would be more efficient than TMZ chemotherapy currently exists and most of the clinical trials have unfortunately failed to demonstrate increased overall survival of patients. Phase III clinical trials in patients with newly diagnosed or recurrent GBM have been reviewed recently (Stepanenko and Chekhonin, 2018). Moreover, molecular biomarkers that would reliably predict response to therapy are largely missing except for the *MGMT* promoter methylation that associates with benefit from chemotherapy in the newly diagnosed GBMs (Le Rhun et al., 2019). Development of targeted therapies has mostly focused on inhibition of aberrant oncogenic signaling, cell-cell communication, and interactions within the microenvironment. Recently, immunotherapy has gained a lot of interest in treatment of cancer. Another interesting area of investigation is drug repurposing aiming at testing already approved drugs for different indications (Le Rhun et al., 2019). Furthermore, efforts to directly target CSCs have mostly failed to demonstrate clinical benefit, whereas targeting the stem cell niche or CSC plasticity represent potential ways to eradicate CSCs (Saygin et al., 2019) (Figure 5). The future treatment of GBM most likely combines different treatment strategies that are individualized according to the precise molecular features of each tumor (Reifenberger et al., 2017).

4.1.1. Targeting oncogenic signaling pathways

Most targeted therapy efforts have focused on aberrant signaling via receptor tyrosine kinases such as EGFR, PDGFRA, MET, and FGFR, and their associated signaling pathways such as PI3K/Akt/mTOR and RAS/MAP kinase that promote tumor cell proliferation and survival (Prados et al., 2015; Le Rhun et al., 2019). EGFR has been widely studied as a therapeutic target since it is the most prominent oncogene in GBM (Brennan et al., 2013). Also, identification of the EGFRvIII, which is a constitutively active oncogenic variant of EGFR arising from deletions of exons 2-7, has been a target of active drug development (Figure 5A) (Prados et al., 2015).

Most studies investigating the potential of small molecule inhibitors targeting EGFR, such as gefinitib and erlotinib, have failed to show efficacy in phase II clinical trials of GBM (Rich et al., 2004; van den Bent et al., 2009; Yung et al., 2010). Other approaches including for example rindopepimut, a peptide-based vaccine targeting the EGFRvIII, failed to increase survival in a phase III trial of patients with newly diagnosed GBM (Weller et al., 2017). Other selected phase II and III clinical trials of

targeted therapies in GBM have been recently reviewed (Le Rhun et al., 2019). Taken together, targeting the oncogenic signaling pathways has not demonstrated efficacy in randomized controlled trials of patients with GBM (Reifenberger et al., 2017).

Disappointing efficacy with small molecule inhibitors demonstrates the challenge of clinical translation. One critical feature is the intratumoral heterogeneity of tumor cells that is characteristic to GBM (Prados et al., 2015). Another important mechanism underlying resistance to small molecule inhibitors is the presence of alternative compensatory signaling pathways (Roth and Weller, 2014). In addition, treatment can drive clonal evolution of the tumor meaning that the proportion of tumor cells responsive to specific targeted therapy can vary, thus complicating the efforts to develop effective therapies (Aldape et al., 2019).

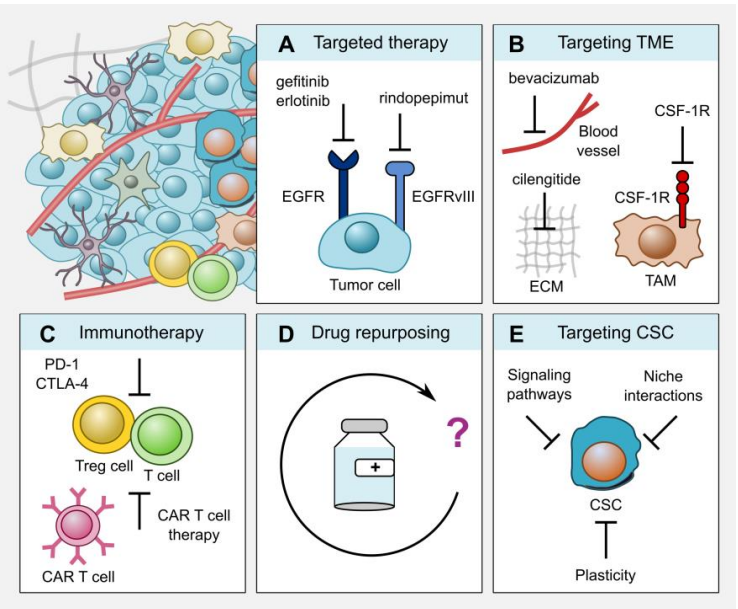

Figure 5. Therapeutic strategies in GBM. Several different strategies have been developed to improve survival of patients with GBM: A) Targeted therapy, B) targeting tumor microenvironment (TME), C) immunotherapy, D) drug repurposing, and E) targeting CSC.

4.1.2. Targeting tumor microenvironment

GBMs are highly vascularized tumors and the vascular supply is essential for tumor growth. VEGF is the central regulator of angiogenesis and has been the key target for development of antiangiogenic therapies. Bevacizumab (Avastin, Roche, Germany) is a humanized anti-VEGFA antibody (Figure 5B) which was demonstrated to improve progression-free survival of patients with recurrent GBM in two phase II clinical trials (Friedman et al., 2009; Kreisl et al., 2009). Based on these promising trials, bevacizumab was approved by the FDA for treatment of recurrent GBM in 2009 (Kim

et al., 2018). Two phase III clinical trials evaluated the benefit of bevacizumab in combination with the standard treatment in patients with newly diagnosed GBM. Unfortunately, both studies failed to improve overall survival of patients (Chinot et al., 2014; Gilbert et al., 2014).

Traditional antiangiogenic therapy aims to block tumor's blood supply by inhibiting formation of new blood vessels and by depleting the existing ones to starve the tumor which would slow down tumor progression and improve patient survival. However, impaired blood perfusion in tumors results in TME that is hypoxic and acidic and has been shown to have several adverse effects. Hypoxia and acidosis fuel tumor progression by promoting tumor cell invasion, metastasis, and immune evasion and increases resistance to radio- and chemotherapy (Jain, 2014). In contrast, lower doses of antiangiogenic therapy might improve patient outcome. This concept is known as vessel normalization which aims to normalize the abnormal tumor vasculature, resulting in improved blood perfusion, decreased hypoxia and more efficient drug delivery to the tumor (Jain, 2014).

Several approaches have been developed to target communication between tumor cells and the tumor microenvironment. Targeting integrins, that are essential for mediating cell adhesion, migration, and invasion, was considered a highly promising strategy to block interactions between tumor cells and the ECM (Figure 5B). However, a phase III clinical trial that investigated the efficacy of a selective integrin inhibitor, cilengitide (Merck EMD 121974, Germany), combined with the standard therapy in patients with GBM failed to improve clinical outcomes of patients (Stupp et al., 2014). Another interesting strategy to inhibit communication between tumor cells and the innate immune system was to inhibit the CSF-1 receptor (CSF-1R), which is expressed on the surface of TAMs (Figure 5B). A selective inhibition of CSF-1R significantly increased survival of animals of a genetically engineered mouse model of proneural GBM and inhibited tumor growth of patient-derived xenografts (Pyonteck et al., 2013). Unfortunately, CSF-1R inhibitor showed no efficacy in phase II clinical trial in patients with recurrent GBM (Butowski et al., 2016).

4.1.3. Immunotherapy approaches

Immunotherapy holds high expectations as a future standard of care for cancer. Ideally immunotherapy harnesses the patient's own immune system to attack tumor cells. As glioma tumor microenvironment is highly immunosuppressive, strategies that exploit activation of antitumor immune responses to overcome immunosuppression have been suggested as potential therapeutic approaches in patients with gliomas. Several different strategies are available including for example vaccination, immune-checkpoint blockade, and chimeric antigen receptor (CAR) T-cell therapy (Reifenberger et al., 2017). For example, a peptide-based vaccination against mutant IDH protein was developed and it induced antitumor immune response in a preclinical study of GBM suggesting that anti-IDH vaccine could have potential as immunotherapy approach in treatment of IDH-mutated GBMs (Schumacher et al., 2014). Alternative approach for vaccination therapy is the dendritic cell vaccines where patient's own dendritic cells, also known as antigen presenting cells, are loaded ex vivo with tumor-associated antigens or RNAs encoding such antigens and then

injected back into patient as an immunotherapy to induce an anti-tumor immune response. One such example study used dendritic cells loaded with cytomegalovirus phosphoprotein 65 (pp65), which is often expressed in GBM but not in normal brain tissue and demonstrated suppressed tumor growth in animals and improved clinical outcomes in a small cohort of patients (Mitchell et al., 2015).

Immune checkpoint inhibitors such as PD-1 and CTLA-4 have proven efficient in other cancers, but their potential benefit in GBM patients is still under investigation (Figure 5C) (Reifenberger et al., 2017). Currently, phase II and phase III clinical trials on PD-1 blockade in combination with standard radiation and/or chemotherapy are ongoing (Broekman et al., 2018). Oncolytic virotherapy has been proposed to increase the effect of immune-checkpoint blockade. In a preclinical study using immunocompetent mouse GBM models, treatment with a combination of oncolytic virus and immune checkpoint inhibitors PD-1 and CTLA-4 was highly effective and eradicated tumors and prolonged the animal survival (Saha et al., 2017). Another immunotherapy approach is the CAR T cell therapy (Figure 5C), which involves adoptive transfer of genetically engineered chimeric antigen receptor against tumor-specific targets such as EGFRvIII. Treatment with CAR T cells directed against EGFRvIII significantly reduced tumor growth in immunocompetent mouse model of glioma (Sampson et al., 2014). However, a recent phase I clinical trials using CAR T cells targeting EGFRvIII failed to demonstrate marked clinical benefit in small cohorts of patients with recurrent GBM (O'Rourke et al., 2017; Goff et al., 2019).

4.2. Drug repurposing

Drug discovery and development is a highly complex and long-lasting process that requires huge investments with low success rates. Due to limited success with current therapies, there is a need to develop more effective and safe drugs for treatment of cancer. Drug repurposing, which gains growing interest, aims to reposition already clinically approved drugs for treatment of a disease(s) other than the original indication (Figure 5D). Major advantages of this approach include reduced time, cost, and risks, because safety and pharmacokinetic profiles of these drugs have been previously characterized (Ashburn and Thor, 2004). Potential drugs under investigation or already tested in clinical trials with a goal to be repurposed in treatment of GBM have been recently reviewed (Basso et al., 2018; Siegelin et al., 2021).

One such example is metformin, which is the most used drug for treatment of type 2 diabetes and has been extensively studied as a possible therapy option in cancer and especially in GBM. Several studies have reported inhibition of cell proliferation and migration of GBM cells as well as induction of apoptotic cell death (Isakovic et al., 2007; Wurth et al., 2013; Seliger et al., 2016). A recent study using pooled data from three randomized clinical trials of patients with newly diagnosed GBM reported that metformin alone or in a combination therapy was not associated with improved overall survival and concluded that additional studies are needed to identify specific tumor characteristics that would indicate potential benefit from treatment with metformin (Seliger et al., 2020).

Another extensively studied and potential drug for repurposing in treatment of GBM is a well-known antimalarial drug chloroquine. Chloroquine is an autophagy inhibitor and can enhance the efficacy of chemotherapy (Basso et al., 2018; Weyerhauser et al., 2018). Studies investigating the combination of TMZ and chloroquine in GBM cells reported reduced cell proliferation and induced apoptotic cell death in response to the treatment (Hori et al., 2015; Lee et al., 2015). A recent study demonstrated that combination treatment with triarylpyridine compounds and chloroquine causes cell death in several different cancer cell lines via induction of lysosomal membrane permeabilization (LMP), which is an alternative pathway leading to apoptotic cell death (Beauvarlet et al., 2020). However, a phase I/II clinical trial studying hydroxychloroquine in combination with radio- and chemotherapy failed to demonstrate significant improvement in overall survival of patients with newly diagnosed GBM (Rosenfeld et al., 2014).

Drugs affecting the nervous system such as antipsychotics, antidepressants, and antihistamines represent a large group of drugs known as cationic amphiphilic drugs (CADs). CADs are well characterized and tolerated drugs with a long clinical history in treatment of various psychiatric disorders and allergies. Due to their ability to cross the BBB, these drugs are being increasingly tested in treatment of cancer and especially GBM (Ellegaard et al., 2016; Siegelin et al., 2021). Interestingly, some epidemiological studies have reported significantly lower incidences of cancer among patients with schizophrenia and allergy compared to the general population (Barak et al., 2005; Chou et al., 2011; Turner, 2012). Antipsychotics typically inhibit dopamine receptors or alternatively block histamine, serotonine, or adrenergic receptors (Siegelin et al., 2021). The phenothiazines, a class of antipsychotics, that antagonize dopamine signaling has been demonstrated to have anti-glioma effect as detected by decreased cell proliferation and induced apoptosis of rat glioma cells. The glioma cells were more sensitive to the treatment compared to primary mouse brain cells (Gil-Ad et al., 2004). Another study demonstrated that combined treatment with quetiapine and TMZ significantly suppressed tumor growth and prolonged animal survival in orthotopic xenografts of murine GBM possibly via differentiation of GSCs into a less aggressive oligodendrocyte-like cells, that sensitized the cells to chemotherapy (Wang et al., 2017).

4.3. Therapeutic targeting of cancer stem cells

A rationale behind targeting CSCs is that although therapies targeting proliferating tumor cells might lead to significant tumor shrinkage, it may allow the survival of CSCs, that are rather resistant to therapies, and sufficient to cause tumor relapse (Zhou et al., 2009). In principle, eradication of CSCs might provide clinical benefit for patients (Suva and Tirosh, 2020). Potential strategies for targeting CSCs include for example targeting stem cell maintenance pathways, CSC-associated cell surface markers, interactions within the stem cell niche, and the plasticity of CSCs (Gimple et al., 2019; Saygin et al., 2019). Selected emerging therapies specifically targeting CSCs that are currently in clinical trials have been recently reviewed (Saygin et al., 2019).

Significant attempts to target CSCs have focused on development of small molecule inhibitors targeting the signaling pathways critical for the maintenance of CSCs

(Figure 5E) such as Notch, Hedgehog, and Wnt pathways (Zhou et al., 2009). Other signaling pathways important for the maintenance of CSCs and thus, potential therapeutic targets are TGF-β, PI3K, janus kinase (JAK)/STAT3, and NF-κB. A major concern with this strategy is that most stem cell signaling pathways in CSCs are shared with normal stem cells, which causes limitations and safety issues about possible toxicity to normal stem cells (Saygin et al., 2019). Cell surface markers expressed by CSCs have been a target for antibody-based therapeutics. However, due to cellular heterogeneity and plasticity of CSCs, the expression of the cell surface markers can vary between patients, tumor types, or even within the same tumor. Since most of the cell surface markers expressed by CSCs are also shared with normal stem cells, finding a potential therapeutic window might be challenging (Zhou et al., 2009).

Targeting the CSC-niche interactions or alternatively CSC plasticity represent potential ways to eradicate CSCs (Figure 5E). Targeting the C-X-C motif chemokine receptor 4 (CXCR4) and the focal adhesion kinase (FAK) with specific inhibitors to block the CSC-niche interaction are currently in phase II clinical trials of patients with hematological malignancies and ovarian cancer, respectively (Saygin et al., 2019). Antiangiogenic therapy represents a potential strategy to indirectly target GSCs via disruption of the perivascular niche where the GSCs typically reside. Interestingly, antiangiogenic treatment with bevacizumab depleted GSCs and suppressed tumor growth in xenograft models of GBM (Bao et al., 2006; Calabrese et al., 2007). Another study investigating the effects of bevacizumab in patient-derived GBM xenografts demonstrated that significant reduction in the tumor blood supply in response to treatment with bevacizumab was, however, accompanied with increased tumor cell invasion caused by induced hypoxia (Keunen et al., 2011).

Targeting CSC plasticity could suppress the tumor growth or sensitize the tumor cells to treatment (Figure 5E). This could be achieved by induction of a specific cell state that is known to be less tumorigenic, less plastic, or more vulnerable for therapeutic targeting (Suva and Tirosh, 2020). However, more studies are needed to better understand the relationship and dynamics between different cell states and their therapeutic potential. A study investigating the therapeutic potential of BMP4 showed that treatment of patient-derived GSCs with BMP4 induced cell differentiation in vitro and treatment of orthotopic patient-derived glioblastoma xenografts with BMP4-loaded heparin acrylic beads in vivo significantly prolonged the survival of animals suggesting that induction of cell differentiation could potentially target the GSCs (Piccirillo et al., 2006). A recent study showed that treatment of patient-derived GBM cells with BMP4 induced specific cell state transitions as demonstrated by lineage-tracing and single-cell RNA-sequencing of barcoded GBM cells (Larsson et al., 2021). Importantly, this new strategy allows measurement of transcriptional states of single GBM cells over time and in response to treatment which could be useful for development of therapies targeting tumor cell plasticity in the future (Larsson et al., 2021).

5. Experimental models to study glioblastoma

It is generally accepted in the field that established classic GBM cell lines, such as U87MG, U251, and T98G that have been extensively passaged in serum-

supplemented growth medium in vitro, do not represent reliable models of GBM (Robertson et al., 2019). Due to long-term culture in the presence of serum, these cells are genetically unstable and tumors that develop upon xenotransplantation have lost their invasiveness and therefore do not resemble GBM (Lee et al., 2006). Moreover, a recent study performed authentication analysis of glioma cell lines and showed that the U87MG distributed by the American Type Culture Collection (ATCC) does not match the original cell line established in Uppsala nearly 50 years ago, suggesting an unknown origin of these cells (Allen et al., 2016).

Patient-derived primary GBM cells provide a disease-relevant model to study GBM. These patient-derived cells are cultured in serum-free medium supplemented with essential growth factors, which enriches the GSCs and cells grow in suspension as gliospheres. The gliosphere cultures are more stable and better recapitulate the genotype and transcriptional state of the parental tumor. Upon orthotopic xenografting into the mice brain, patient-derived gliospheres are highly tumorigenic and more closely recapitulate the growth characteristics and tumor cell heterogeneity present in the original human GBM (Lee et al., 2006). These patient-derived xenografts (PDX), however, propagate in immunodeficient mice, resulting in the tumor microenvironment that is significantly different from the actual microenvironment and more importantly devoid of the immune system (Linkous et al., 2019).

Brain organoids and brain slice cultures provide more complex ex vivo models. Brain organoids are simplified laboratory-grown 3D cultures typically derived from human embryonic stem cells or induced pluripotent stem cells (Lancaster et al., 2013; Linkous et al., 2019). Protocols for patient-derived GBM organoids have also been recently established (Linkous et al., 2019; Jacob et al., 2020). GBM organoids recapitulate the cellular heterogeneity present in the original tumor and are highly tumorigenic when transplanted into the animal brain but lack certain essential interactions with the microenvironment. GBM organoids are suitable for example for biobanking, xenograft studies, and high-throughput drug screenings, which likely facilitates the development of personalized therapies (Jacob et al., 2020). An advantage of the brain slice cultures is the possibility to investigate cell-cell interactions between tumor cells and host cells and to perform microscopic imaging in real time (Robertson et al., 2019).

Other approaches to model GBM are to use genetically engineered mouse models (GEMMs) or syngeneic mouse models. GEMMs with specifically engineered mutations in oncogenes such as *PDGFR* and *EGFR* or tumor suppressor genes such as *NF1* to drive spontaneous tumor formation have widely increased our understanding of the genetic drivers of GBM tumorigenesis (Hambardzumyan et al., 2011). As the tumor formation in GEMMs is spontaneous and cannot be controlled, it can be time consuming with some animal-to-animal variation. In syngeneic mouse models for example carcinogen-induced murine glioma cells are transplanted back into mouse brains to induce tumor formation in syngeneic hosts. The commonly used GL261 murine glioma model does not, however, genetically, or histologically resemble an authentic GBM (Robertson et al., 2019). Both GEMMs and syngeneic mouse models have the important advantage of modeling cellular and immune interactions in their native environment. On the other hand, murine tumors do not fully recapitulate human tumors. Taken together, none of these experimental models is a

perfect representation of human GBM and inefficient improvements in treatment of GBM may have suffered from the limitations of the clinically relevant study models. Advances in gene editing will likely facilitate the development of relevant models (Aldape et al., 2019).

6. Novel glioblastoma-associated biomarkers

6.1. Mammary-derived growth inhibitor

Mammary-derived growth inhibitor (MDGI), also known as the heart-type fatty acid binding protein (H-FABP/*FABP3*) is a small 13-kDa cytoplasmic protein expressed at high levels at least in the heart, skeletal muscle, kidney, lung, and brain tissues (Veerkamp et al., 1990). At least nine different FABPs have been identified and named according to the tissue where they were first identified, although their expression is more ubiquitous (Glatz and van der Vusse, 1996; Borchers et al., 1997). H-FABP was purified and characterized from rat heart tissue (Fournier et al., 1978; Said and Schulz, 1984) while MDGI was originally purified from the bovine mammary gland. The molecular mass was predicted to be between 12-14 kDa and MDGI was reported to inhibit proliferation of mammary carcinoma cells and was therefore named as mammary-derived growth inhibitor (Bohmer et al., 1984).

In a following study, MDGI was reported to have extensive sequence homology to the FABPs and retinoic acid-binding proteins with the highest homology with the rat H-FABP (Bohmer et al., 1987). Moreover, based on biochemical studies, mammary FABPs were reported to be very similar to the FABP isolated from the rat heart (Jones et al., 1988). Finally, these studies led to a conclusion that H-FABP and MDGI are functionally indistinguishable (Clark et al., 2000).

MDGI inhibits cell proliferation and promotes differentiation of the normal mouse mammary epithelial cells (Yang et al., 1994). In addition to growth inhibitory function, MDGI is involved in intracellular transport of fatty acids (FAs) and lipid metabolism. Most FABPs bind long-chain fatty acids with high affinity and exhibit additional preference towards polyunsaturated fatty acids (PUFAs) (Glatz and van der Vusse, 1996; Richieri et al., 2000). In addition to FA binding properties, FABPs have been proposed to be involved in the transport of lipids to specific cellular compartments. FABPs transport lipids for example to lipid droplets for storage, to the endoplasmic reticulum for membrane biosynthesis and signaling, to the mitochondria for energy production, and to the nucleus for regulation of gene expression (McKillop et al., 2019). Studies in H-FABP knock-out animals demonstrated elevated levels of long-chain FAs in plasma indicating impaired uptake and utilization of FAs by cardiac myocytes (Binas et al., 1999; Schaap et al., 1999).

MDGI becomes overexpressed in several types of cancer, but regulation of its expression as well as its role in cancer remains controversial. MDGI expression in gastric cancer associated with tumor aggressiveness, metastasis, and poor patient survival (Hashimoto et al., 2004). MDGI expression was significantly increased in metastatic uveal melanomas compared to non-metastatic tumors (Linge et al., 2012; Crabb et al., 2015). In non-small cell lung cancer, high MDGI expression predicted

poor survival supporting tumor promoting function for MDGI (Tang et al., 2016). MDGI has been suggested, however, to function as a tumor suppressor in breast cancer. Overexpression of MDGI in human breast cancer cells reduced cell proliferation, colony formation and tumorigenicity in vivo, suggesting a tumor suppressor function for MDGI in breast cancer (Huynh et al., 1995). A tumor suppressor role was further supported by a study demonstrating that MDGI expression is downregulated in breast cancer cell lines and primary breast tumors by hypermethylation (Huynh et al., 1996). Moreover, MDGI was reported to inhibit integrin activity via direct interaction with the cytoplasmic tails of several integrin α-subunits, which suppressed invasion of breast cancer cell lines (Nevo et al., 2010). In glioma cells, MDGI expression was induced by hypoxia in a HIF-1α-dependent manner and MDGI-mediated FA uptake and lipid droplet formation were essential for cell growth and survival under hypoxia (Bensaad et al., 2014). Our research group previously identified overexpression of MDGI in tumor cells and tumor-associated vasculature of orthotopic GBM xenografts and clinical glioma samples (Hyvonen et al., 2014).

6.2. CD109

CD109 is a 180-kDa glycoprotein of the α2-macroglobulin/complement family expressed at the cell surface of activated T cells and platelets, hematopoietic stem and progenitor cells, and endothelial cells (Sutherland et al., 1991; Murray et al., 1999; Lin et al., 2002). CD109 is bound to the cell surface via its C-terminal glycosylphosphatidylinositol (GPI)-anchor. CD109 is synthesized as a 155-kDa core protein that after glycosylation and maturation in the endoplasmic reticulum and the Golgi complex becomes a 205-kDa glycoprotein. The mature CD109 is cleaved in the trans-Golgi network into 180-kDa and 25-kDa subunits by furin and finally expressed at the cell surface as a complex of two subunits (Hagiwara et al., 2010). The large 180-kDa CD109 can be released from the cell surface and is capable of thioester-mediated covalent binding whereas the small 25-kDa GPI-anchor containing subunit remains bound to the plasma membrane (Lin et al., 2002; Hagiwara et al., 2010).

CD109 was characterized as a TGF-β co-receptor that negatively regulates the TGF-β signaling pathway in human keratinocytes in vitro (Finnson et al., 2006). A follow-up study demonstrated that CD109 promoted internalization and degradation of the TGF-β receptor as a regulatory mechanism to inhibit activation of the TGF-β signaling (Bizet et al., 2011). Moreover, treatment of human keratinocytes with soluble recombinant CD109 downregulated TGF-β signaling, but at the same time upregulated STAT3 phosphorylation and Bcl-2 expression, which increased cell survival of human keratinocytes in vitro (Litvinov et al., 2011). *CD109* knockout animals were generated to study the physiological function of CD109 in vivo. *CD109*-deficient mice were viable with only minor defects in the hair growth. A closer histological analysis revealed abnormalities in the skin, especially hyperplasia of the epidermis in the *CD109*-deficient animals was evident compared to the wild-type animals. Interestingly, increased levels of STAT3 phosphorylation were reported in the *CD109*-deficient animals whereas no effect on TGF-β signaling was observed (Mii et al., 2012). Despite these important findings that describe CD109 as a regulator of

various signaling pathways, the physiological function of CD109 remains still largely unknown.

CD109 is also highly expressed in several cancers and has been recently widely studied in the context of cancer. High expression of CD109 has been detected in several cancer cell lines including skin, lung, and esophageal squamous cell carcinomas and GBM whereas most normal tissues showed no CD109 expression (Hashimoto et al., 2004). High CD109 has also been reported in surgically resected human lung tumors (Sato et al., 2007), basal-like breast carcinomas (Hasegawa et al., 2008), and brain tumors (Shiraki et al., 2017). In a previous study from our research group, a proteomic analysis of biotinylated cell surface proteins was performed to analyze differentially expressed proteins between metastatic and non-metastatic isogenic tumor cell lines. Several cell surface proteins were identified to be overexpressed in the metastatic cell lines including CD109 (Karhemo et al., 2012). More recently, CD109 expression was reported to be associated with poor survival of patients with lung cancer and CD109 was demonstrated to promote lung cancer metastasis via a signaling mechanism involving activation of the JAK/STAT3 pathway (Chuang et al., 2017).

AIMS OF THIS STUDY

GBM is among the deadliest cancers and remains a therapeutic challenge with practically no cure. The aim of this study was to elucidate mechanism underlying GBM tumor initiation and progression, identify vulnerabilities as possible novel targets for therapies, and test the efficacy of such targeted therapies in relevant preclinical models of GBMs.

Specific aims of this study were the followings:

i. To study the functional roles and molecular mechanisms of the GBM-associated proteins MDGI and CD109 in tumor initiation and progression

ii. To investigate the potential of an antihistamine clemastine as a lysosome-targeted therapy in a preclinical study of glioblastoma

iii. To understand the mechanisms how CD109 maintains the glioblastoma cell stemness

MATERIALS AND METHODS

The most important materials and methods used in this study are briefly described here. Detailed description can be found in the original publications (I, II).

1. Materials

1.1. Cell lines

Table 1. The following cell lines were used in this study.

Cell line	Origin	Source	Used in
BT3	Human GSCs	Generated in the lab	I, II
BT3 CD133+*	Human GSCs	Generated in the lab	II
BT5	Human gliosarcoma cells	Generated in the lab	I
BT5R	Human recurrent gliosarcoma/GBM cells	Generated in the lab	I
BT11	Human GSCs	Generated in the lab	I, II
BT12	Human GSCs	Generated in the lab	I, II
BT13	Human GSCs	Generated in the lab	I, II
BT18	Human GSCs	Generated in the lab	II
ZH161	Human GSCs	Prof. M. Weller, Zürich	I
ZH305	Human GSCs	Prof. M. Weller, Zürich	I, II
S24	Human GSCs	Prof. M. Weller, Zürich	I, II
LN229	Human GBM cells	Prof. M. Weller, Zürich	I
LN308	Human GBM cells	Prof. M. Weller, Zürich	I
U87MG	Human GBM cells	ATCC	I
293FT	Human embryonic kidney cells	ATCC	I, II
bEnd.3	Immortalized murine brain endothelial cells	ATCC	I
HuAR2T	Immortalized human umbilical vein endothelial cells	Prof. P. Ojala, Helsinki	I
iNHA	Immortalized human brain astrocytes	Lonza	I
H2	Human GBM, giant cell variant	Prof. S. Meri, Helsinki	II

* BT3 CD133+ is a FACS-sorted CD133+ cell population derived from the parental BT3.

1.2. Reagents

Table 2. The most important reagents used in this study.

Reagent	Description	Reference	Used in
Cathepsin B substrate	Enzyme activity measurement	Calbiochem	I
Clemastine fumarate	LMP induction	Sigma-Aldrich	I

Fibrinogen	3D culture matrix	Calbiochem	II
Fugene 6	Transfection reagent	Promega	I, II
Gefinitib	EGFR inhibition	InvivoGen	I
K777	Pan-Cathepsin inhibitor	AdipoGen Life sciences	I
Laminin	Coating of surface for cell adhesion	Invitrogen	II
LLOMe*	LMP induction	Santa Cruz	I
Methylcellulose stock solution	Colony forming assay	R&D Systems	I, II
MTT*	Cell viability measurement	Sigma-Aldrich	I, II
Poly-D-Lysine	Coating of surface for cell adhesion	Sigma-Aldrich	I, II
rhCD109	Recombinant human CD109	R&D Systems	II
rhEGF	Recombinant human EGF	Peprotech	I, II
rhFGF-basic	Recombinant human FGF-basic	Peprotech	I, II
rhIL-6	Recombinant human IL-6	Peprotech	II
Stattic	STAT3 inhibitor V	Sigma-Aldrich	II
Temozolomide	DNA alkylating agent	MedChemExpress	II

* LLOMe: L-leucyl-L-Leucine O-methyl ester; MTT: Thiazolyl Blue Tetrazolium Bromide.

1.3. Lentiviral shRNA-constructs

Table 3. Lentiviral shRNA-constructs in pLKO.1 vector.

Lentivirus	Description	Construct ID	Used in
ShControl	Non-targeting control	SHC002	I, II
shMDGI#1	Human *FABP3* silencing	TRCN0000059680	I
shMDGI#2	Human *FABP3* silencing	TRCN0000059681	I
shCD109#1	Human *CD109* silencing	TRCN0000073649	II
shCD109#2	Human *CD109* silencing	TRCN0000073648	II
shGP130#1	Human *IL6ST* silencing	TRCN0000058287	II
shGP130#2	Human *IL6ST* silencing	TRCN0000058285	II

1.4. Antibodies

Table 4. Primary antibodies and antibody conjugates used in this study.

Antigen	Source	Reference	Used in
α-SMA Cy3 conjugate	Mouse monoclonal	Sigma-Aldrich	II
β-actin	Rabbit polyclonal	Cell Signaling Technology	I
β-tubulin	Mouse monoclonal	BD Pharmingen	I, II
Annexin V conjugate	Alexa Fluor™ 488	Molecular Probes	I
pAkt (Thr308)	Rabbit polyclonal	Cell Signaling Technology	I

Akt	Rabbit polyclonal	Cell Signaling Technology	I
BAD	Mouse monoclonal	Santa Cruz Biotechnology	I
Calnexin 1	Mouse monoclonal	Santa Cruz Biotechnology	I
Caspase-3	Rabbit monoclonal	Cell Signaling Technology	I
Cleaved Caspase-3 (Asp175)	Rabbit monoclonal	Cell Signaling Technology	I, II
CD31	Rat monoclonal	BD Pharmingen	I, II
CD109	Sheep polyclonal	R&D Systems	II
CD109 (C-9)	Mouse monoclonal	Santa Cruz Biotechnology	II
CD109	Mouse monoclonal	R&D Systems	II
CD109 11H3	Mouse monoclonal	IBL America	II
Collagen IV	Rabbit polyclonal	Sigma-Aldrich	II
pEGFR (Tyr1068)	Rabbit polyclonal	Cell Signaling Technology	I
EGFR	Rabbit polyclonal	Santa Cruz Biotechnology	I
pERK1/2 (Thr202/Tyr204)	Mouse monoclonal	Cell Signaling Technology	I
ERK1	Rabbit polyclonal	Santa Cruz Biotechnology	I
Galectin-1	Rabbit polyclonal	Abcam	I
GAPDH	Mouse monoclonal	Europa Bioproducts	I
GP130 (E-8)	Mouse monoclonal	Santa Cruz Biotechnology	II
GP130	Goat polyclonal	R&D Systems	II
HIF-1α	Rabbit polyclonal	Cell Signaling Technology	I
Ki-67	Mouse monoclonal	Dako	II
Lamin A/C	Rabbit monoclonal	Abcam	II
LAMP2	Rabbit polyclonal	Abcam	I
MDGI	Mouse monoclonal	Santa Cruz Biotechnology	I
MDGI	Rat monoclonal	R&D Systems	I
NG2	Rabbit polyclonal	R&D Systems	II
NUMA Cy3 conjugate	Mouse monoclonal	Sigma-Aldrich	I, II
Olig2	Goat polyclonal	R&D Systems	II
p27	Rabbit polyclonal	Santa Cruz Biotechnology	I
pP53 (Ser15)	Rabbit polyclonal	Cell Signaling Technology	I
P53	Mouse monoclonal	Santa Cruz Biotechnology	I
PDGFRα	Mouse monoclonal	R&D Systems	II
PDGFRβ	Rabbit polyclonal	Santa Cruz Biotechnology	II
Phalloidin-TRITC* (F-actin)	Peptide	Sigma-Aldrich	II
Podocalyxin	Rat monoclonal	R&D Systems	I, II
SOX2	Goat polyclonal	R&D Systems	II
pSTAT3 (Tyr705)	Rabbit polyclonal	Cell Signaling Technology	II
STAT3	Mouse monoclonal	Cell Signaling Technology	II
Vimentin Cy3 conjugate	Mouse monoclonal	Sigma-Aldrich	I, II

* TRITC: Tetramethylrhodamine B isothiocyanate, a fluorescently labelled peptide that stains filamentous Actin (F-Actin).

2. Methods

2.1. Cell culture (I, II)

The cell lines and their origin used in this study are described in Table 1. All the patient-derived GBM cell lines (referred as BT) were maintained as gliosphere cultures in serum-free DMEM/F-12 medium supplemented with 1x B-27 supplement, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 15 mM HEPES, 0.02 µg/ml EGF, and 0.01 µg/ml FGF-basic (Table 2). ZH161, ZH305, and S24 were cultured in Neurobasal medium supplemented with 1x B-27 minus vitamin A supplement, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.02 µg/ml EGF, and 0.02 µg/ml FGF-basic (Table 2). LN229, LN308, and the commercial U87MG and bEnd.3 cell lines were cultured according to manufacturer's instructions in DMEM 1.0 g/l glucose supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The commercial 293FT cell line was cultured according to manufacturer's instructions in DMEM 4.5 g/l glucose supplemented with 10% FBS, 2mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. H2 cell line was cultured in RPMI-1640 medium supplemented with 10% FBS, 2mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Immortalized human umbilical vein endothelial cells (HuAR2T) were maintained in endothelial cell growth medium 2 (EBM2) supplemented with growth medium 2 supplement pack (PromoCell) and 2.0 µg/ml doxycycline. Immortalized normal human astrocytes (iNHA) were maintained in astrocyte basal medium (ABM) with SingleQuots supplement pack (Lonza). All the cell lines were maintained at +37°C in a humidified incubator with 5% CO_2.

2.2. Cell stimulations and treatments (I, II)

For serum-induced cell differentiation experiments, GSCs were dissociated as single cells and plated in complete DMEM medium supplemented with 10% FBS for indicated times (II). For hypoxia treatment, cells were incubated in complete growth medium O/N and then in a hypoxia chamber (Ruskinn Technology Limited) in the presence of 1% of O_2 and 5% CO_2 for 24h (I). To demonstrate that clemastine induces LMP, several GSCs or normal cells (iNHA, HuAR2T, 293FT, and bEnd.3) (Table 1) were treated with 1µg/ml of clemastine fumarate (Sigma-Aldrich) or DMSO O/N at +37°C followed by immunofluorescence staining with the galectin-1 antibody (I). For experiments studying the activation of STAT3 signaling pathway, CD109-silenced or non-targeted control GSCs were cultured in complete growth medium without growth factors O/N at +37°C and then stimulated with recombinant human IL-6 (50 ng/ml) for 15 and 30 min (Table 2). The cells were then lysed and analyzed by Western blotting (II).

2.3. Lentiviral-shRNA vectors (I, II)

The specific shRNA constructs in pLKO.1 vector were obtained from the RNAi Consortium shRNA library (Broad Institute of MIT and Harvard) (Table 3). To

produce the lentivirus, the specific shRNA-containing plasmid or non-targeting control plasmid (scrambled) and the lentiviral packaging plasmids pCMVg and pCMVΔ8.9 (Addgene) were co-transfected into the 293FT cells (Table 1) by using the Fugene 6 transfection reagent (Promega). The lentiviral supernatants were collected 48h and 72h after transfection and sterile filtered. Target cells were transduced with the lentiviral supernatant together with 8 μg/ml of Polybrene (Sigma-Aldrich) for O/N. The silencing efficiency was assessed by qRT-PCR and/or Western blot analysis. Experiments were started at day 4-6 post-transduction. Complete sequences of the shRNA constructs are provided in the respective original publication (I, II).

2.4. Western blot analysis (I, II)

For preparation of the protein extracts, cells were first washed with ice-cold PBS and then lysed in SDS-lysis buffer containing 150 mM Tris-HCl (pH 6.8), 1.2% SDS, 30% glycerol, and 15% β-mercaptoethanol or in RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, and 0.5 mM DTT together with protease and phosphatase inhibitors (both from Roche) for 30 min on ice. The samples were cleared by microcentrifugation at 14 000g for 20 min at +4°C (I). In the study II, the cells were lysed in RIPA buffer containing 150 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 1% SDS, 2% octyl-β-D-glucopyraniside, 0.5 mM DTT together with protease and phosphatase inhibitors. The protein extracts were sonicated and then cleared by microcentrifugation as described above. Protein concentrations of the cell extracts were measured by using the Pierce BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The samples were boiled in 2 x Laemmli sample buffer + 5% β-mercaptoethanol. Equal amounts of protein per sample (10-20 μg) were separated on Tris-Glycine Mini Gels (Invitrogen, Thermo Fisher Scientific) and transferred to PVDF membranes using the Trans-Blot Turbo Transfer system (Bio-Rad Laboratories). The membranes were blocked with 5% non-fat dry milk or 5% bovine serum albumin (BSA) in TBS with 0.1% Tween 20 (TBS-T) at RT for 1h and then incubated with appropriate primary antibodies (Table 4) O/N at +4°C. The membranes were washed and subsequently incubated with the horseradish peroxidade (HRP)-conjugated secondary antibodies (Dako) for 30 min at RT. After washes, the signal was detected by using the SuperSignal West Pico ECL substrate (Thermo Fisher Scientific) or Clarity Western ECL substrate (Bio-Rad Laboratories) and exposed to FUJIFILM Super RX-N.

2.5. Immunoprecipitation (II)

For the immunoprecipitation assay, cells were first washed with ice-cold PBS and then lysed in IP-lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 1 μg leupeptin together with protease and phosphate inhibitors (Roche). The samples were lysed for 5 min on ice and then microcentrifuged at 14 000g for 10 min at +4°C. Protein concentrations were determined using a Pierce

BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The lysates were precleared for 30 min at RT by incubating with pre-washed Dynabeads Protein G (Thermo Fisher Scientific). Then 700 µg of protein was used for immunoprecipitation together with 7.5 µg of anti-GP130 antibody (Santa Cruz Biotechnology) (Table 4) for O/N at +4°C. Appropriate IgG control antibody served as a negative control. The immunocomplexes were incubated with pre-washed Dynabeads Protein G magnetic beads for 1.5 h at RT, then pulled down, washed several times, and finally eluted in 4 x Laemmli sample buffer (Bio-Rad Laboratories). Samples were further analyzed by Western blotting as described above.

2.6. qRT-PCR (I, II)

Total RNA was isolated from cells using the Nucleospin RNA II kit (Macherey-Nagel). 1.0 µg of RNA was used for cDNA synthesis via reverse transcription using the iScript cDNA synthesis kit (Bio-Rad Laboratories) according to manufacturer's instructions. qRT-PCR was performed using KAPA SYBR fast qPCR master mix (2X) (Kapa Biosystems) and the CFX96 real-time PCR detection system (Bio-Rad Laboratories). The human ADP-ribosylation factor 1 (*ARF1*) was used as a housekeeping gene to normalize the gene expression levels. Relative gene expression levels were quantified by using the Delta Ct method (ΔCt). Complete primer sequences are provided in the respective original publication (I, II).

2.7. MTT and drug sensitivity assays (I, II)

MTT assay was used to measure cell proliferation and viability in vitro. Cells ($5x10^3$) in 100 µl of complete growth medium were plated in 3-10 replicates in 96-well plates. At the indicated time points, 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml in PBS) (Table 2) was added to the cells. The plates were incubated for 2h at +37°C. Cells were then lysed with 10% SDS and 10 mM HCl in H_2O for O/N at +37°C. The absorbances were measured at 540 nm using the FLUOstar Omega microplate reader (BMG Labtech).

For the EGFR inhibition experiments (I), GSCs were treated with 0.1-10 µM of gefinitib (InvivoGen) (Table 2) for 48h followed by cell viability measurement by the MTT assay. For the pan-cathepsin inhibition studies (I), the cells were treated with 0, 1, or 2 µM of K777 (pan-cathepsin inhibitor) (AdipoGen Life Sciences) (Table 2). At indicated time points, cell viability was measured by the MTT assay. Fresh K777 inhibitor was added every other day. For measurement of clemastine cytotoxicity (I), the cells were treated with 1 µg/ml, 2 µg/ml, or 5 µg/ml of clemastine fumarate (Sigma-Aldrich) (Table 2). At indicated time points, the cell viability was measured by the MTT assay as described above. For TMZ sensitivity experiments (II), the cells were treated with 250 µM of TMZ (MedChemExpress) (Table 2) for four days. Fresh TMZ was added after two days of culture. Cells treated with DMSO served as negative controls. The cell viability was measured by the MTT assay as described above. For a combination therapy with Stattic (specific STAT3 inhibitor, Sigma-Aldrich) (Table 2) and TMZ (II), the cells were first treated with 1 µM of Stattic for O/N and then with

250 μM of TMZ for an additional two days. Cells treated with DMSO served as negative controls. The cell viability was measured by the MTT assay as described above

2.8. Cell viability and apoptosis assays (I, II)

Cell viability was assessed either by using trypan blue exclusion method (study I) or CellTiter-Glo luminescent cell viability assay (study II). Briefly, for determination of cell viability by the trypan blue assay, cells were dissociated as single cells, diluted 1:2 in 0.4% trypan blue (Sigma-Aldrich), and counted. Cell viability was determined based on the dye uptake: clear cytoplasm (viable cells) versus blue cytoplasm (nonviable cells). The cell viability formula: (number of viable cells / total number of cells) x 100. Viability of cells or colonies embedded in the 3D fibrin matrix was measured based on the released ATP levels. At indicated time points, CellTiter-Glo reagent (Promega) was mixed 1:1 with complete growth medium in a 96-well plate. The plate was mixed vigorously for 5 min at RT and incubated for 25 min at RT to stabilize the luminescent signal. Finally, the luminescent signal was measured by using the FLUOstar Omega microplate reader (BMG Labtech). Cellular apoptosis was determined by a flow cytometric assay based on Annexin V staining (study I). Annexin V staining identifies apoptosis by binding to phosphatidylserine residues exposed at the outer cell membrane at early stages of apoptosis. Single cell suspensions of control and MDGI-silenced GSCs were stained with Annexin V, Alexa Fluor™ 488 conjugate (Thermo Fisher Scientific) (Table 4) for 15 min at RT. Cells were washed with binding buffer containing 10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$ and then fixed with 4% PFA containing 2.5 mM $CaCl_2$ for 10 min at +4°C. Cells were suspended into the binding buffer and the fluorescence signal was detected by flow cytometry (BD Accuri C6). Data analysis was performed using FlowJo v10.1 software (Tree Star Inc.).

2.9. Colony forming assays (I, II)

For a colony forming assay in semi-solid methylcellulose matrix, $1x10^4$ control or shRNA-silenced patient-derived GSCs were suspended as single cells in complete growth medium supplemented with the growth factors and then mixed with methylcellulose stock solution (R&D Systems) (Table 2). The final concentration of methylcellulose was 1.3%. The mixtures were carefully applied on 35-mm cell culture plates in triplicates using a syringe and a needle. The cultures were incubated for three weeks at +37°C with 5% CO_2. At the experimental endpoint, the colonies were imaged using Nikon Eclipse Ti-E inverted microscope. Quantification of the colonies was performed by using the CellProfiler image analysis software (http://cellprofiler.org/citations/). For soft agar colony forming assay, $1x10^3$ cells were suspended in complete growth medium containing 0.35% agarose. The mixtures were added on top of a 2-ml layer of pre-solidified 0.7% agar in complete medium in 6-well plates. Complete growth medium was added twice weekly. At indicated time points, the colonies were imaged using inverted Zeiss Axiovert 200 microscope. Quantification of the colonies was performed by using the ImageJ software (National Institute of Health, USA).

2.10. Immunofluorescence staining of cells (I, II)

Cells were first detached as single cell suspensions and then plated on coverslips and incubated for O/N at +37°C. Patient-derived GSCs that grow as gliospeheres were plated on coverslips coated with poly-D-lysine (Sigma-Aldrich) (Table 2). Coverslips were washed with PBS, fixed with 4% PFA in PBS for 10 min at RT, and then permeabilized with 0.5% NP-40 in PBS or 0.3% Triton-X100 in PBS for 5 min at RT. After washes with PBS the coverslips were blocked with 3% BSA in PBS or in 10% FBS containing 0.03% Triton-X100 in PBS and incubated with appropriate primary antibodies (Table 4) for 1h at RT. The coverslips were then incubated with specific fluorescence-conjugated secondary antibodies (Alexa Fluor, Thermo Fisher Scientific) for 30 min at RT. Finally, after washes with PBS, the coverslips were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories).

2.11. Analysis of lysosomal membrane permeabilization (I)

LMP can be visualized in cells by a galectin puncta-staining assay that has been described previously (Aits et al., 2015). For analysis of LMP in non-targeted control and MDGI-silenced patient-derived GSCs, the cells were plated on poly-D-lysine-coated (Table 2) coverslips as single cells and incubated for O/N at +37°C. Cells were stained according to the immunofluorescence staining protocol of cells described above using a specific antibody detecting galectin-1 (LGALS1, Abcam) (Table 4). GSCs treated with 2 mM of L-leucyl-L-leucine O-methyl ester (LLOMe, Santa Cruz Biotechnology) (Table 2) for 4h to induce LMP served as a positive control. To demonstrate that clemastine induces LMP, several GSCs and normal cells were treated with 1µg/ml of clemastine fumarate (Table 2) for 24h at +37°C followed by immunofluorescence staining with the anti-galectin-1 antibody (Table 4). Cells treated with DMSO served as a negative control. The coverslips were scanned for digital image analysis using a 3DHISTECH slide scanner. The cytoplasmic galectin puncta-staining was quantified using Panoramic viewer software (3DHISTECH). Representative microscopic images were acquired with Zeiss LSM 880 confocal microscope.

2.12. Cytoplasmic cathepsin B activity (I)

A protocol for measurement of cytoplasmic cathepsin B activity was obtained and modified from (Jaattela and Nylandsted, 2015). Briefly, patient-derived GSCs ($2x10^4$) in 100 µl of complete growth medium with growth factors were plated in 96-well plate coated with poly-D-lysine (Sigma-Aldrich) (Table 2) as four replicates and incubated for O/N at +37°C. Cytosolic and total cell extracts were prepared by lysing the cells in lysis buffer containing digitonin at concentrations of 20 µg/ml and 200 µg/ml, respectively. Cells were lysed on ice for 15 min after which the extracts were collected and transferred on new 96-well plates. To measure the cathepsin B enzyme activity, 50 µl of cytosolic and total cell extracts were mixed with 50 µl of cathepsin B substrate (Calbiochem) (Table 2) diluted in reaction buffer. After incubation for 5 min at +30°C, the kinetics of cathepsin B activity was measured using the FLUOstar Omega

microplate reader (BMG Labtech). The cathepsin B enzyme activity in the cytosol was calculated by normalizing the measured values to the total protein concentration of the corresponding well, determined by the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific), and then by comparing to the measured total cellular activity.

2.13. Mass spectrometric analysis of lysosomal extracts (I)

Lysosomal extracts were prepared from non-targeted control and MDGI-silenced patient-derived GSCs at 6d post-transduction by using the Lysosome Enrichement Kit for Tissue and Cultured cells (Thermo Fisher Scientific) according to the manufacturer's instruction. To obtain enough sample material, six independent cultures were pooled together for one readout. After the lysosomal extraction by gradient ultracentrifugation, the purity of the collected sample fractions was confirmed by Western blot analysis using specific antibody against lysosomal protein LAMP2 (Table 4). Extracted lysosomal pellets were stored at -80°C until mass spectrometric analysis. For mass spectrometric analysis, lipids were extracted from the lysosomal extracts and identified and quantified by direct infusion electrospray ionization-tandem mass spectrometry using Agilent 6490 Triple Quad LC/MS with iFunnel technology (Agilent Technologies Inc.) at the Helsinki University Lipidomics Unit. A more detailed protocol of the mass spectrometric analysis is provided in the respective original publication (I).

2.14. 3D fibrin cultures (II)

For the 3D fibrin cultures, 6 mg/ml of fibrinogen (Calbiochem) (Table 2) was dissolved in Hank's Balanced Salt Solution (HBSS) for 2h at +37°C. Patient-derived GSCs ($3x10^3$) transduced with control or CD109 targeting shRNAs were suspended in 15 µl of fibrinogen. Polymerization was initiated by addition of 15 µl of Thrombin (4 U/ml, Sigma-Aldrich) and 15 µl of Aprotinin (400 µg/ml, Sigma-Aldrich) in HBSS to the mixtures. The mixtures were pipetted on 24-well plates and allowed to polymerize for 1h at +37°C. Complete growth medium supplemented with growth factors and Aprotinin (100 µg/ml) were added to the cultures. Fresh growth medium and the supplements were added twice a week. Cell growth in the 3D fibrin matrix was followed by microscopic imaging using EVOS FL inverted epifluorescence microscope (Thermo Fisher Scientific). The 3D fibrin cultures were fixed with 4% PFA in PBS for 1h at RT and then stored at +4°C in PBS until immunofluorescence stainings.

2.15. Immunofluorescence staining of 3D fibrin cultures (II)

Before the immunofluorescence staining, the 3D fibrin cultures were post-fixed in ice-cold acetone-methanol (1:1) followed by washes with PBS. The 3D fibrin cultures were then blocked in 15% FBS and 0.3% Triton X-100 in PBS for 2h at RT. After washes with PBS, the 3D fibrin cultures were then incubated with appropriate primary antibodies (Table 4) for O/N at +4°C. The 3D fibrin cultures were washed extensively

first with 0.45% Triton X-100 in PBS and then with PBS followed by incubation with specific fluorescence-conjugated secondary antibodies (Alexa Fluor, Thermo Fisher Scientific) for 4h at RT. After washing with PBS, the 3D fibrin cultures were finally mounted with Vectashield containing DAPI. Zeiss AxioImager.Z1 upright epifluorescence microscope equipped with Apotome and Hamamatsu Orca R2 1.3-megapixel monochrome CCD camera was used for imaging.

2.16. Proximity ligation assay (II)

Proximity ligation assay (PLA) was performed using the Duolink In Situ Red Starter Kit (Mouse/Goat; MilliporeSigma) and according to the manufacturer's instructions. Patient-derived GSCs were plated as single cells on Laminin-coated (1 μg/cm^2; Invitrogen) (Table 2) Lab-Tek II chamber slides (Nunc, Thermo Fisher Scientific) and incubated O/N at +37°C. Cells were fixed with 4% PFA in PBS for 10 min at RT and then blocked with a specific blocking reagent for 1h at RT. Cells were incubated with specific primary antibodies against CD109 (mouse monoclonal, R&D Systems), CD109 11H3 (mouse monoclonal, IBL America), and GP130 (goat polyclonal, R&D Systems) (Table 4) diluted 1:200 in the antibody diluent for O/N at +4°C. Subsequently steps including PLA probe incubation, ligation, and amplification were performed according to the manufacturer's instructions. Slides were mounted in mounting medium with DAPI, provided in the kit, and imaged using a Zeiss LSM880 confocal microscope. To study whether the soluble CD109 can interact with GP130, CD109-silenced patient-derived GSCs were treated with 500 ng/ml of recombinant CD109 (R&D Systems) for 4h prior the fixation and analysis of the PLA signal as described above.

2.17. Tumor microarrays (I, II)

MDGI expression was analyzed in glioma tumor microarrays obtained from surgically operated patients at the Helsinki University Hospital (I). Low grade glioma tumor microarrays were prepared from tumor biopsies of 112 patients and has been described previously (Puputti et al., 2006). GBM tumor microarray consisted of 36 tumor biopsies and has been described previously (Joensuu et al., 2005). For immunohistochemical analysis, formalin-fixed paraffin-embedded (FFPE) tissues were deparaffinized and rehydrated and stained using the Tyramide Signal Amplification kit (TSA) kit (Perkin Elmer) according to the manufacturer's instructions. The antigen was retrieved in a citrate buffer (1.8 mM citric acid, 8.2 mM sodium citrate, pH 6.0) with heat treatment. Endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol and non-specific binding was blocked with a TNB blocking buffer for 30 min. The sections were incubated with an anti-MDGI antibody (R&D Systems) (Table 4) O/N at +4°C followed by incubation with biotinylated secondary antibody (Dako) and then with HRP-conjugated streptavidin. The specific signal was amplified by using a biotinylated tyramide and HRP-conjugated streptavidin. The staining was visualized with 3-amino-9-ethylcarbatzole (AEC). Finally, the sections were counterstained with Mayer's hematoxylin and mounted.

Clinical patient material for evaluation of CD109 expression was obtained from surgically operated patients at the Tampere University Hospital. Tumor classification and grading was performed by a neuropathologist according to the WHO criteria published in 2007 (Louis et al., 2007). The tumor microarrays consisted of 385 primary human astrocytomas and 120 recurrences. The CD109 immunostaining protocol was optimized for Lab Vision Autostainer 480 (Thermo Fisher Scientific) using the HRP/DAB detection system (Dako). FFPE sections (5 µm) were deparaffinized and antigen was retrieved in Tris-HCl (pH 8.5) with heat treatment using a Lab vision PT-module (Thermo Fisher Scientific). The tumor microarray slides were blocked with Dako REAL antibody diluent followed by incubation with an anti-CD109 antibody (R&D Systems) (Table 4) diluted 1:400 in the Dako REAL antibody diluent for 1h. The slides were then incubated with a specific HRP-conjugated secondary antibody (Dako) and the staining was visualized with the Dako REAL DAB+ substrate. Finally, the slides were counterstained with Mayer's hematoxylin, mounted and scanned for digital image analysis using a 3DHISTECH slide scanner.

2.18. Ex vivo organotypic culture (I)

Five- to eight-week-old female FVB mice were anesthetized using ketamine and xylazine and the brains were collected. The cerebellum was excised into two hemispheres, which were then embedded in 4% low melting point agarose (Thermo Fisher Scientific) in PBS. The brain tissue was cut into 500 µm thick sections using a vibratome (HistoLab). Brain tissue slices were incubated on a 0.4-µm filter membranes (Millipore) placed in 6-well plates containing complete Neurobasal A medium (Gibco) supplemented with 1 mM L-glutamine, 1x B-27, and 1% penicillin/streptomycin at +37°C with 5% CO_2. Fresh culture medium was supplied every other day to the tissue slice cultures. Gliospheres were formed from the stably transfected GFP or MDGI-GFP expressing U87MG cells by incubating $4x10^3$ cells in 0.6% agarose-coated 96-well plates with U-bottom O/N at +37°C. The gliospheres were carefully transferred on the brain tissue slices and their growth was followed ex vivo by imaging at indicated time points using Leica TCS SP2 confocal microscope.

2.19. In vivo intracranial xenografts (I, II)

Six-week-old immunocompromised Rj:NMRI-Foxn1nu/Foxn1nu (Janvier Labs) mice were anesthetized using a mixture of ketamine and xylazine or under 2.5% isoflurane and placed on a stereotaxic injector (World Precision Instruments). Patient-derived GSCs ($1x10^5$) in 5 µl of PBS were intracranially injected at +2 mm right, -1 mm anteroposterior from the bregma, and at +2.5 mm depth. Post-operative analgesia (temgesic) was administered locally for an additional two days. For clemastine preclinical study, tumor cells were intracranially transplanted as described above and after two weeks of tumor formation animals were randomized into two cohorts: vehicle and clemastine. Clemastine was administered intraperitoneally for 12 days at a dose of 100 mg/kg on the first day followed by 50 mg/kg daily. For the survival study, clemastine treatment was continued until physical signs of tumor burden. PBS

was administered intraperitoneally as a control in the vehicle group. At the experimental endpoints, animals were sacrificed, the brain tissues were collected, and snap-frozen in -50°C isopentane (Honeywell). Tissue samples were stored at -80°C until processing and immunofluorescence stainings.

2.20. Immunofluorescence staining of xenograft tissue sections (I, II)

Snap-frozen brain tissues were cut into 9 µm thick coronal sections by using a cryotome (Leica CM1950). For immunofluorescence staining the tissue sections were thawed, then washed with PBS, and fixed in 4% PFA in PBS for 20 min at RT. Tissue sections were then blocked with 5% FBS and 0.03% Triton X-100 (MilliporeSigma) in PBS for 1h at RT and then incubated with appropriate primary antibodies for O/N at +4°C. Tissue sections were washed with 0.03% Triton X-100 in PBS and then incubated with specific Alexa Fluor-conjugated secondary antibodies (Thermo Fisher Scientific) for 1h at RT. After several washes, nuclei were counterstained with DAPI and the tissue sections were mounted with Mowiol mounting medium (MilliporeSigma). The slides were scanned for digital image analysis using a 3DHISTECH slide scanner.

2.21. RNA sequencing (II)

Total RNA was extracted from the patient-derived GSCs using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. RNA quality was confirmed by using a Tapestation (Agilent). Total RNA (10 ng) was used for preparation of cDNA libraries for the next-generation sequencing by using NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs). Finally, RNA was sequenced by using an Illumina NextSeq500 High Output (1x75bp) sequencer (Functional Biology Unit, Finland) followed by data processing and data analysis. A more detailed protocol of the RNA sequencing is provided in the respective original publication (II).

2.22. Ethics statement (I, II)

The use of clinical patient material for tumor microarrays were approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa (study I) and Tampere University Hospital (study II). The use of glioma tissue biopsies for generation of patient-derived cell lines was approved by the ethics committee of the Pohjois-Savo Health Care District municipalities (53/2009). Before the study, the written informed consent was obtained from all the patients. All animal experiments were approved by the National Animal Experiment Board in Finland and Regional State Administrative Agency of Southern Finland. All animal experiments were performed under a license (ESAVI/6285/04.10.07/2014 and ESAVI/403/2019) and in accordance with Finnish legislation regarding animal experiments.

2.23. Statistical analyses (I, II)

Data are representative of at least three independent experiments unless stated otherwise. Data are presented as mean ± standard deviation or ± standard error mean, as indicated. Fisher's exact test and Chi-squared tests were used to analyze distribution of categorical variables. Log-rank test was used to compare survival distributions of the groups. Unpaired two-tailed student's t-test or nonparametric Mann-Whitney U-test was used to compare means between two groups. Analysis of multiple comparisons were performed by using one-way ANOVA with the Kruskal-Wallis test, or two-way ANOVA with Tukey's or Sidak's multiple comparisons tests. For all statistical tests P values < 0.05 were considered statistically significant. Statistical analyses were performed with the IBM SPSS (22.0) and GraphPad Prism (8.0) softwares.

RESULTS AND DISCUSSION

1. Profiling transcriptome and tumorigenic potential of patient-derived GSCs (I, II)

Patient-derived GSCs represent a widely accepted model to study GBM. Unlike traditional serum-cultured GBM cell lines, GSC cultures are more stable in vitro and better recapitulate the growth pattern present in the original human GBM such as invasive growth pattern and tumor cell heterogeneity upon xenotransplantation into the animal brain (Lee et al., 2006). Then, in order to have a comprehensive set of patient-derived GSCs covering all GBM subtypes, we generated stable GSC cultures from surgical samples of several patients with high grade gliomas (Le Joncour et al., 2019). GSCs grew as gliospheres in serum-free culture conditions, expressed GSC markers such as SOX2 and Olig2, and were able to form colonies from single cells as studied by colony forming assays. These GSCs were also able to differentiate into astrocytic-like cells for example in the presence of serum and importantly, were highly tumorigenic upon intracranial injection into the brain of immunocompromised mice. This functional characterization demonstrated that GSCs generated in the lab fulfill the functional criteria of GSCs, and therefore represent a relevant model to study GBM (Lathia et al., 2015).

Interestingly, each GSC line displayed a distinct and characteristic tumor growth pattern in vivo that was highly reproducible between the experiments. Two major and distinct phenotypes were identified: formation of a tumor bulk with varying degree of invasion and satellite tumor formation as well as activation of angiogenesis or alternatively highly infiltrative growth pattern with vessel co-option but without a clear tumor bulk or activation of angiogenesis. These observed tumor phenotypes correspond to recently demonstrated growth patterns of mesenchymal (MES) and proneural (PN) clinical GBM subtypes, respectively (Jin et al., 2017).

We profiled the GSCs by performing a bulk RNA-sequencing and gene expression analysis. We compared the gene expression profiles to the recently published gene signatures (Neftel et al., 2019). Neftel and colleagues demonstrated that single GBM cells exist in four main cell states: MES-like, AC-like, OPC-like, and NPC-like. Moreover, they compared the cell state signatures to different GBM subtypes and found that enriched MES and AC-like cell state signatures corresponded to the MES and CL GBM subtypes, respectively.

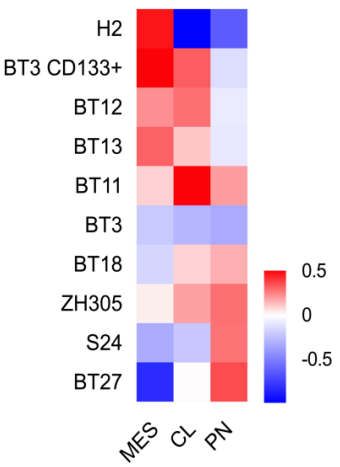

Figure 6. Classification of patient-derived GSCs and the H2 cell line. The cell lines were classified based on their dominant transcriptional subtype as follows: MES-like, CL-like, and PN-like. The figure has been modified from Filppu et al., 2021 with the permission from the American Society for Clinical Investigation.

The PN GBM subtype was enriched with OPC and NPC-like cell state signatures (Neftel et al., 2019). Based on the gene expression analysis, our GSCs typically displayed enrichment of two or more cell states which is consistent with the previous studies (Patel et al., 2014; Neftel et al., 2019). We classified our GSCs based on the dominant cell state as follows: H2, BT3 CD133+, BT13, and BT12 as MES-like, and BT18, ZH305, S24, and BT27 as PN-like (Figure 6). We observed that the MES-like signature typically co-occurred with the AC-like signature. Finally, BT11 and BT3 cells were classified as hybrids as they displayed mixed phenotypes. Importantly, the transcriptomic profiles supported the observed in vivo growth patterns of the GSCs. The MES-like BT12 and BT13 formed a large tumor bulk with varying levels of invasion and activation of angiogenesis. The growth pattern of the PN-like BT18, ZH305, and S24 GSCs was highly infiltrative with vessel co-option. These observations were supported by a recent study (Jin et al., 2017).

2. MDGI and CD109 both associate with tumor progression and poor survival (I, II)

In a previous study from our research group, MDGI was discovered to be expressed in human clinical glioma samples in a grade-dependent manner as well as in the tumor-associated vasculature in addition to the tumor cells (Hyvonen et al., 2014). To further study the possible clinical relevance of MDGI in gliomas, we performed immunohistochemical analysis of two cohorts of tumor microarrays consisting of low-grade gliomas (LGG) and GBMs. These analyses were supported by investigation of *MDGI* mRNA levels in several publicly available datasets all accessed via the GlioVis dataportal (Bowman et al., 2017).

As expected, positive MDGI staining was observed in the tumor cells and in tumor-associated endothelium. Evaluation of the MDGI staining intensities revealed positive correlation with CD117/C-Kit receptor in GBM that has been previously shown to be expressed in the endothelium of perinecrotic and hypoxic regions of GBM (Sihto et al., 2007). We found no significant association between MDGI expression and EGFR or EGFRvIII expression, EGFR phosphorylation (Tyr-1173), or p53 in the tumor microarrays. Moreover, *FABP3*/MDGI mRNA levels did not associate with *IDH1* mutation status in the TCGA LGG dataset.

In the tumor microarray cohort consisting of LGGs, moderate to high MDGI expression significantly associated with poor survival and MDGI was an independent prognostic factor. This finding was supported by the analysis of the TCGA dataset for LGGs and GBMs (TCGA GBMLGG) which demonstrated that *FABP3* mRNA levels significantly correlated with increasing tumor grade and poor survival. *FABP3*/MDGI did not however associate with patient survival in GBMs alone neither in the GBM tumor microarray cohort nor in the TCGA GBM dataset. This finding is possibly explained by the very short overall survival of patients with GBM. Interestingly, analysis of the Ivy_Gap dataset showed that the highest *FABP3* expression was in the areas of leading edge and infiltrative tumor cells suggesting a possibility that MDGI would promote glioma cell invasion.

To investigate the expression of CD109 in clinical glioma samples, we analyzed a large cohort of clinical glioma tumor microarray specimens (grades 2-4) by immunohistochemistry. Positive CD109 immunostaining located at the cell surface of tumor cells was observed in most specimens. Evaluation of the CD109 staining intensities revealed that expression of CD109 significantly associated with increasing tumor grade. CD109 was not significantly associated with *IDH1* mutation, p53, or EGFR expression. Interestingly, CD109 significantly associated with STAT3 phosphorylation (pSTAT3, Tyr705) and increased number of Ki-67 positive tumor cells. A distinct statistical analysis of LGGs (grades 2 and 3) and GBMs (grade 4) alone showed a significant correlation between CD109 and pSTAT3 or Ki-67 only in GBMs suggesting that the CD109-STAT3 association would be specific to GBM. Moreover, CD109 expression associated with significantly poorer survival of patients with grade 2-4 gliomas.

To link our results with publicly available datasets, we studied the *CD109* mRNA level expression in multiple datasets using the GlioVis dataportal (Bowman et al., 2017). Consistent with the tumor microarray results, high *CD109* mRNA levels in the TCGA LGG and TCGA GBMLGG datasets significantly associated with poorer survival whereas such association was not detected in the TCGA GBM dataset alone. A recent study suggested that *CD109* expression is associated with the MES GBM subtype in the TCGA GBM dataset (Minata et al., 2019). To confirm this finding, we analyzed *CD109* mRNA expression in a total of 17 individual GBM datasets available in the GlioVis dataportal. The *CD109* mRNA levels were highest in the MES subtype tumors and lowest in the PN subtype tumors and the result was consistent between different datasets. The MES subtype of GBM has been previously linked with increased immune cell infiltration (Wang et al., 2017). STAT3 has also been previously linked to MES subtype and poor patient survival in GBM (Carro et al., 2010) thus supporting our findings. CD109 has been demonstrated to promote lung cancer metastasis via activation of STAT3 signaling pathway (Chuang et al., 2017) whereas in GBM the association between CD109 and STAT3 pathway has remained unclear. In a study investigating CD109 in glioma progression, association between CD109 and pSTAT3 was not observed in a genetically engineered mouse model of glioma between tumors that were developed in control or *CD109*-deficient mice (Shiraki et al., 2017). Taken together, these results demonstrate that both MDGI and CD109 are clinically relevant markers for GBM aggressiveness although with different functions.

3. Targeting lysosomal membrane integrity by antihistamine impairs glioblastoma invasion and prolongs animal survival in a preclinical study (I)

Invasive GBM cells are the major clinical challenge in treatment of GBM. These therapeutically resistant and invasive cells cannot be targeted with current therapies and cause frequent relapse of the tumor. In the study I, we utilized clinical patient samples, patient-derived GSCs, high-throughput lipidomics analyses, and preclinical in vivo xenograft models to explore the nature of invasive GBM cells. Our results demonstrate that MDGI increases glioma cell invasion in vivo and revealed a novel role for MDGI in the maintenance of lysosomal membrane integrity. Moreover,

MDGI silencing induced cell death via induction of LMP. Importantly, we identified LMP as a novel vulnerability of invasive GBM cells that could be targeted by antihistamines for therapeutic interventions. Interestingly, our results support repurposing of LMP inducing agents such as antihistamine clemastine in treatment of invasive GBMs.

3.1. MDGI overexpression promotes glioblastoma invasion

A previous study demonstrated that MDGI expression in GBM cells is induced by hypoxia, which subsequently increased fatty acid uptake and lipid storage and promoted cell survival (Bensaad et al., 2014). Analysis of several GSC cultures and established long-term GBM cell lines revealed that GSCs co-expressed MDGI and HIF-1α whereas the GBM cell lines expressed neither. Expression levels of MDGI and HIF-1α were induced in the GBM cell lines by hypoxia. Moreover, GSCs that grow as gliospheres were already hypoxic as demonstrated by detectable HIF-1α expression which likely explains why MDGI was also upregulated in the GSC cultures. Thus, these results confirm the previous finding that hypoxia induces MDGI expression in a HIF-1α-dependent manner (Bensaad et al., 2014).

Several studies have demonstrated that hypoxia promotes GBM cell invasion (Zagzag et al., 2006; Joseph et al., 2015; Qiu et al., 2019). To study the possibility that MDGI would promote glioma invasion, we overexpressed MDGI as a GFP-fusion protein (MDGI-GFP) in the U87MG GBM cell line. The U87MG cells express low levels of MDGI and upon intracranial implantation form a large non-invasive bulky tumor mass. Our in vitro studies demonstrated that MDGI-GFP overexpression in the U87MG GBM cells did not affect cell proliferation, but significantly increased anchorage independent growth in a colony forming assay compared to the GFP-expressing control cells. Moreover, MDGI-GFP-overexpression induced an invasive growth pattern in an organotypic slice culture model ex vivo compared to the GFP-expressing control cells. Finally, intracranial implantation of GFP-expressing control and MDGI-GFP-overexpressing U87MG cells demonstrated that tumors derived from the MDGI-overexpressing cells displayed significantly more invasive growth pattern with formation of secondary tumors compared to controls that grew bulky tumors as expected. Similar results were obtained with another MDGI-GFP overexpressing GBM cell line LN229.

The role of MDGI in cancer is not well understood and remains somewhat controversial. Both tumor suppressive and tumor promoting functions for MDGI have been suggested depending on the cancer type (Hashimoto et al., 2004; Nevo et al., 2010). Our results support that MDGI promotes invasion and tumor progression in GBMs. Moreover, we and others have demonstrated that MDGI becomes upregulated by hypoxia in a HIF-1α-dependent manner (Bensaad et al., 2014). Since GBMs are highly hypoxic tumors and hypoxia promotes glioma cell invasion, we may speculate that hypoxia would be a mediator of the MDGI's invasion promoting function in GBM.

3.2. MDGI silencing induces caspase-independent apoptosis

To further investigate the functional role of MDGI in GBM, we silenced MDGI expression by using lentiviral-mediated shRNAs. Silencing efficiencies of two *FABP3*-targeting shRNAs were confirmed at mRNA and protein levels. MDGI silencing induced rapid and irreversible morphological alterations by disrupting the gliosphere formation in the GSC cultures and significantly inhibited cell proliferation. Colony forming assays demonstrated that MDGI silencing significantly compromised self-renewal and cell growth of GSCs compared to non-targeted control cells. The reduced colony formation was not linked to loss of stemness, but rather substantial loss of cell viability. Indeed, a cell viability assay demonstrated markedly reduced cell viability of MDGI-silenced GSCs compared to non-targeted control cells. Upon intracranial injection, MDGI-silenced GSCs failed to form tumors whereas the non-targeted control GSCs were highly tumorigenic. To summarize, MDGI silencing unexpectedly induced a dramatic loss of cell viability in GSCs and compromised their tumorigenicity in vivo.

As an indicator of apoptosis, we analyzed annexin V-binding to non-targeted control and MDGI-silenced GSCs by a flow cytometric analysis. Annexin V binds to phosphatidylserine exposed at the surfaces of apoptotic cells (Koopman et al., 1994). The flow cytometric analysis demonstrated that MDGI silencing significantly increased annexin V-binding when compared to non-targeted control GSCs suggesting that MDGI silencing induces apoptotic cell death. We considered EGFR as a potential inducer of apoptosis, since its inhibition has been demonstrated to induce apoptosis of glioma cells and MDGI has been previously linked to EGFR trafficking (Nevo et al., 2009; Ghildiyal et al., 2013). MDGI silencing decreased EGFR expression both at protein and mRNA levels and GSCs expressing high levels of EGFR were highly sensitive to its inhibition by gefitinib. However, GSCs that expressed low levels of EGFR were resistant to gefitinib inhibition but were as sensitive to cell death induced by MDGI silencing as the EGFR-expressing GSCs, suggesting that cell death was independent of EGFR expression. Moreover, we investigated the levels of several pro- and anti-apoptotic proteins in response to MDGI silencing. Importantly, we did not detect an increase in the expression of cleaved caspase-3, which is a mediator of caspase-mediated apoptosis. From these experiments, we concluded that MDGI silencing induced cell death of GSCs that was independent of EGFR expression or caspases.

3.3. MDGI silencing induces lysosome-mediated cell death due to significant alterations in the lipid composition of their membranes

The finding that MDGI-induced apoptosis was caspase-independent led us to study alternative pathways leading to apoptosis. Lysosomal cell death induced by LMP is an alternative apoptosis pathway. LMP causes leakage of lysosomal hydrolases such as cathepsin proteases into the cytosol and depending on the extent of the leakage induces cell death. LMP can be dependent or independent of caspases (Aits and Jaattela, 2013). Several upstream mechanisms leading to LMP have been identified and reviewed by Aits and Jäättelä. These include for example lysosomotropic detergents, proteases, reactive oxygen species, bacterial toxins, viral proteins, and

some lipids and their metabolites (Aits and Jaattela, 2013). LMP can be visualized in cells with the galectin puncta-staining assay, where localization of diffuse cytoplasmic galectin-1 staining changes into a punctate pattern in response to LMP (Aits et al., 2015).

To study LMP induction in GSCs, we first treated the GSCs with L-Leucyl-L-Leucine O-methyl ester (LLOMe), a lysosomotropic detergent and a known chemical inducer of LMP (Aits et al., 2015), and detected punctate staining pattern of galectin-1 as an indicator of LMP. Interestingly, we detected similar change in the galectin-1 staining pattern in response to MDGI silencing in GSCs. Co-staining with antibodies detecting lysosome-associated membrane protein 2 (LAMP2) and galectin-1 verified the lysosomal localization of galectin-1 punctate staining. Moreover, increased cathepsin B activity was measured in cytoplasmic extracts of MDGI-silenced GSCs compared to non-targeted controls suggesting leakage of lysosomal enzyme into the cytosol thus further verifying that the cell death caused by MDGI silencing was mediated by LMP. The MDGI-induced LMP was extensive since the observed cell death was only partially attenuated by treatment with a pan-cathepsin inhibitor. Taken together, we identified LMP as a novel mechanism of MDGI silencing-induced glioma cell death.

MDGI has been previously demonstrated to bind with high affinity to long-chain FAs and especially PUFAs (Richieri et al., 2000). To understand the mechanism underlying MDGI silencing-induced cell death, we analyzed the lipid composition of lysosomal membranes isolated from non-targeted control and MDGI-silenced GSCs by mass spectrometry. High-throughput lipidomics analyses demonstrated marked compositional differences in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) lipid classes. Interestingly, within both PC and PE lipid classes, the proportions of diunsaturated lipid species decreased in response to MDGI silencing while the proportions of monounsaturated lipid species increased. This finding is significant, because PC and PE are the most abundant lipid classes that together comprise about 70% of the phospholipids of the lysosomal membranes (Kobayashi et al., 2002). Similar trend was also observed within the ceramide class. The proportions of monounsaturated ceramide species decreased whereas the unsaturated ceramides increased after MDGI silencing. Collectively, we observed a trend towards lower abundance of lipid saturation, which was likely due to impaired uptake of PUFAs into cells. PUFAs are essential fatty acids that contain more than one double bond in their backbone. Mammalian cells can only produce certain FAs whereas others, particularly PUFAs, must be taken up from external sources (Bi et al., 2020). A previous study demonstrated that in the MDGI knockout animals, total phospholipid mass in the brain was reduced by 17% with the highest decrease in the levels of PUFAs choline glycerophospholipids and arachidonic acid, suggesting reduced incorporation of PUFAs into the lipid membranes (Murphy et al., 2005).

Impaired lipid uptake alters the balance between lipid uptake and de novo lipid synthesis. In response to impaired lipid uptake, mammalian cells activate de novo lipid synthesis, which produces saturated and mono-unsaturated fatty acids (MUFAs) increasing the relative levels of more saturated lipids compared to PUFAs (Ntambi, 1999; Rysman et al., 2010). Membrane lipid saturation has been shown to protect cancer cells from free radicals and chemotherapeutics. On the other hand, it has been demonstrated that saturated lipids pack more densely and changes in the levels of lipid

saturation alter the dynamics of the lipid membranes (Rysman et al., 2010). As demonstrated previously, double bonds in the membrane lipids play a central role in controlling membrane fluidity and its elastic properties (Pan et al., 2009; Bruno et al., 2013). Therefore, increased levels of MUFAs produce more rigid membranes that are prone to leak. Ceramides have also been shown to control membrane permeability by altering the biophysical properties of the membranes. Although the molecular mechanism is poorly understood, ceramide-induced membrane permeabilization could be explained, at least to some extent, by the packing defects (Petersen et al., 2010; Artetxe et al., 2017). We concluded from the lipidomic analyses that impaired FA uptake, particularly essential PUFAs, significantly decreased the elastic properties of the lysosomal membranes, which promoted their leakage and demonstrated an essential role for MDGI in the maintenance of lysosomal membrane integrity.

Lipid synthesis and FA uptake not only regulate synthesis and maintenance of the biological membranes but are also essential for cell metabolism and cancer cell growth since lipids serve as an important energy source and precursors for signaling molecules (Snaebjornsson et al., 2020). Cancer cells modulate their nutrient uptake and metabolism in a process known as metabolic reprogramming to support their growth and survival (Venneti and Thompson, 2017). Lipid metabolism is emerging as an important metabolic alteration in cancer (Snaebjornsson et al., 2020). Glioma cells have increased intracellular stores for lipids due to enhanced synthesis and uptake (Venneti and Thompson, 2017). Hypoxia plays a central role in metabolic reprogramming of cancer cells. In a recent study, HIF-1α promoted lipid uptake and transport and increased de novo synthesis of FAs via upregulation of adipophilin in colorectal cancer cells. Modulation of fatty acid metabolism by oroxylin A, inactivated the Wnt signaling pathway, promoted cell cycle arrest, and suppressed the growth of human colon carcinoma xenografts (Ni et al., 2017). In a preclinical study of prostate cancer, suppression of FA uptake by monoclonal fatty acid translocase (CD36) antibody reduced tumor growth in PDX models (Watt et al., 2019). Moreover, HIF-1α enhanced FA uptake and accumulation of lipid droplets which increased glioma cell survival (Bensaad et al., 2014). Treatment of glioma spheroids in the collagen matrix with an essential PUFA, gamma-linolenic acid, increased tumor cell proliferation and invasion (Bell et al., 1999). Hoang-Minh et al. characterized recently fast-cycling and slow-cycling GBM cells with distinct metabolic needs and therapeutic implications. Interestingly, they demonstrated that slow-cycling tumor cells were infiltrative and drug-resistant, had increased lipid contents, and utilized oxidative phosphorylation instead of glycolysis as their main energy source. Moreover, slow-cycling GBM cells were vulnerable to lipid metabolism blockade by pharmacologic inhibition or genetic targeting of FABP7 (Hoang-Minh et al., 2018). Collectively, these studies demonstrate the importance of lipid metabolism in cancer progression, invasion, and drug resistance.

3.4. Antihistamine treatment eradicates invasive glioblastoma cells in vivo

Several drugs have been identified as inducers of LMP among which is a group of CADs that form a large well-tolerated and characterized group of drugs commonly used to treat psychiatric disorders and allergies. Antihistamines and specifically CADs have been suggested to be repurposed for treatment of cancer (Ellegaard et al., 2016;

Faustino-Rocha et al., 2017). CADs were previously reported to induce LMP in non-small cell lung cancer cells (NSCLC) and to sensitize multidrug resistant NSCLC, breast, and prostate cancer cells to chemotherapy (Ellegaard et al., 2016). Among the CADs we chose clemastine fumarate, which is an FDA-approved first-generation antihistamine used to relieve allergy symptoms by blocking the H1 histamine receptor. Clemastine has a low molecular weight and high lipid solubility, and it crosses the BBB. It is well tolerated despite the typical sedation effect and fatigue that is typical for the first-generation antihistamines (Schran et al., 1996; Faustino-Rocha et al., 2017). Clemastine is currently under a clinical trial for multiple sclerosis (Green et al., 2017). A recent study investigating the potential of clemastine in treatment of myelination disorder in rats in vivo confirmed that clemastine penetrated the BBB, but unfortunately, failed to improve the phenotype of the disease or myelination in the CNS. Importantly, daily treatment with clemastine (10-30 mg/kg) was well-tolerated in animals and caused neither change in neurological phenotype nor induced apoptotic cell death in the brain. Higher doses (20 and 30 mg/kg) were reported to induce transient sedative effect in animals (Turski et al., 2018).

Treatment of several GSCs and normal cell lines with varying concentrations of clemastine in vitro, revealed that GSCs were markedly more sensitive to clemastine treatment compared to immortalized normal human astrocytes (iNHA), endothelial cells (HuAR2T), and embryonic kidney cells (293FT) or murine brain endothelial cells (bEnd.3). Treatment of cells with 1μM of clemastine for three days induced approximately 50% cell death among GSCs without significant cytotoxicity to the normal cells. To verify that clemastine-induced cytotoxicity was mediated by LMP, the cells were treated with 1μM of clemastine overnight followed by immunofluorescence staining with the galectin-1 antibody. In accordance with the cytotoxicity tests, galectin-1 puncta staining was detected in clemastine-treated GSCs, but not in normal cells suggesting that GSCs are more sensitive to LMP induction compared to the normal cells.

To investigate the preclinical potential of clemastine treatment in vivo, we generated orthotopic intracranial xenografts derived from three different GSCs with distinct tumor growth patterns. Daily treatment with clemastine (100 mg/kg on the first day followed by 50 mg/kg) or vehicle were started after 15 days of tumor growth with a duration of 12 days. No adverse side effects were observed except transient fatigue of the animals following clemastine administration as anticipated based on the previous report (Schran et al., 1996). Histological analyses of the xenograft tissues revealed that in the non-invasive MES BT13 model, clemastine treatment did not affect the size of the primary tumor bulk whereas in the MES BT12 model, which displays a multimodal growth pattern (Figure 7A), clemastine treatment significantly reduced the number and distance of invasive tumor cells (Figure 7B) and decreased the number of secondary tumors and vessel co-option. Finally, in the PN ZH305 model that grows as a highly infiltrating pattern (Figure 7A), clemastine treatment completely abolished the tumor growth (Figure 7A and C).

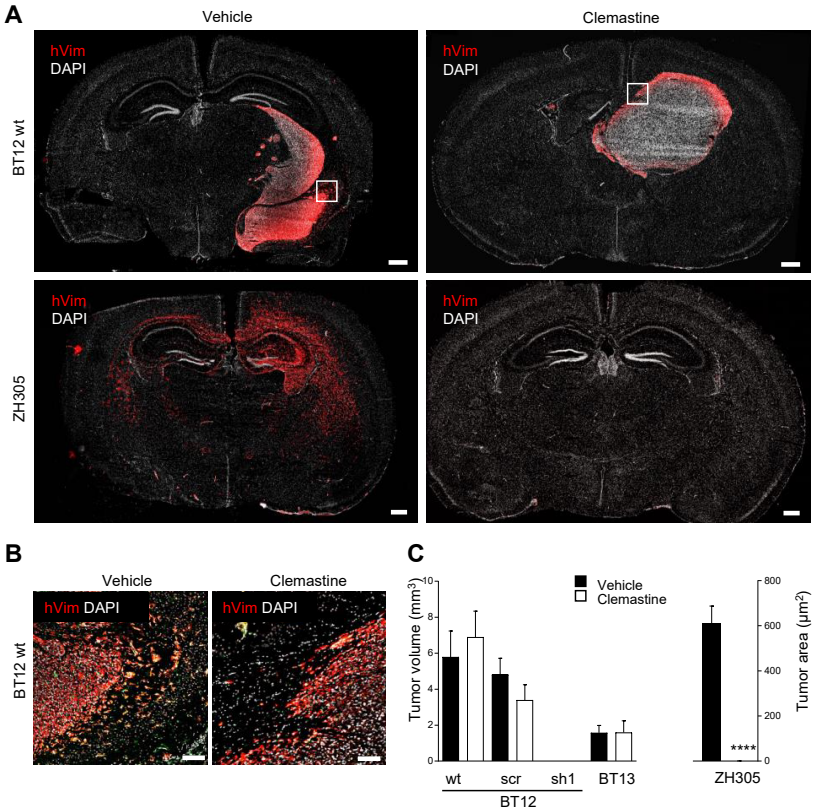

Figure 7. Clemastine treatment eradicates the invasive GBM cells in vivo. A) Representative images of BT12 and ZH305 xenografts after treatment with clemastine or vehicle. B) Higher magnification images of the invading GBM cells in BT12 xenografts after treatment with clemastine or vehicle. Tumor cells were visualized with vimentin (red) and nuclei were counterstained with DAPI (white) in both A and B. Scale bars 500 µm. C) Quantification of the tumor volume/area of the xenograft tumors after treatment with clemastine (white) or vehicle (black). The figure is modified from Le Joncour and Filppu et al., 2019 with the permission from Wiley.

Clemastine treatment induced a significant apoptotic cell death in the xenograft tumor cells as detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). In a survival study, clemastine treatment significantly prolonged the animal survival compared to the vehicle group. Potential explanation why clemastine had no significant effect on the size of the tumor bulk could be the aberrant and poorly functional blood vessels that hamper the delivery of clemastine to the tumor or alternatively the sheltering effect of stromal cells. Considering the potential of lysosomes as therapeutic targets (Appelqvist et al., 2013) and based on our results, we suggest repurposing of clemastine with a new indication and propose clemastine as an adjuvant therapy in treatment of patients with invasive GBMs (Figure 8).

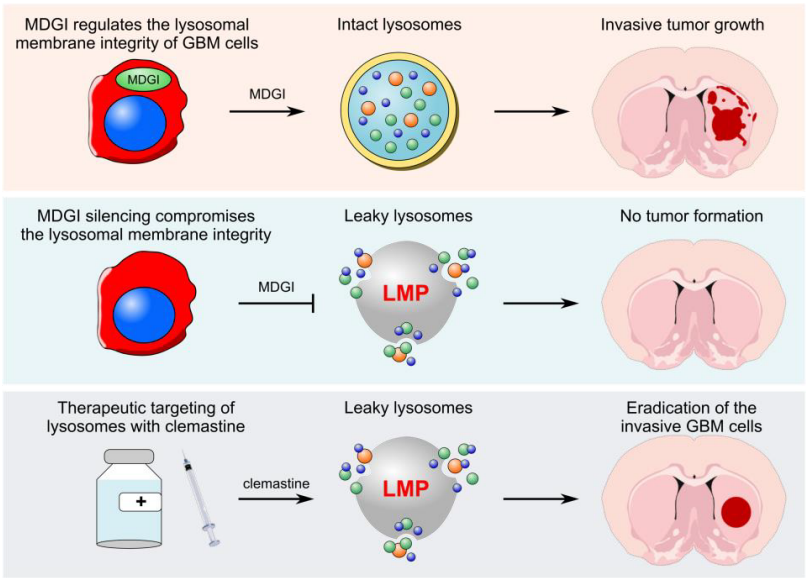

Figure 8. Schematic illustration of the regulation of lysosomal integrity in glioblastoma. MDGI promotes invasive growth of GBM and maintains the lysosomal membrane stability of tumor cells. Silencing of MDGI expression induced GBM cell death via LMP and impaired tumor growth. LMP can be pharmacologically induced in GBM cells by clemastine. Treatment of intracranial xenografts with clemastine eradicated the invasive tumor cells. The schematic illustration is modified from Le Joncour and Filppu et al., 2019.

3.5. Lysosomes as therapeutic targets in cancer

The results of the study I raised an important question, why cancer cells display increased sensitivity to LMP compared to normal cells and more widely what is the potential of lysosome-targeted therapy in treatment of cancer? Lysosomes have been increasingly acknowledged as active organelles involved in various cellular processes and maintenance of cell homeostasis instead of only sites of macromolecule degradation (Appelqvist et al., 2013). Central to the lysosome function are hydrolases such as cathepsins and lysosomal membrane-associated proteins. Interestingly, cancer cells are highly dependent on lysosomes and their effective function and lysosomal alterations are typical in cancer cells. For example, increased lysosomal biogenesis and changes in the lysosomal membrane composition, increased hydrolase activity, and enhanced secretion of the hydrolases have been reported (Kallunki et al., 2013). Changes in the cancer cell lysosomes can promote tumor progression. For example, cancer cells typically have increased expression of cathepsins and the secretion of these proteolytically active enzymes outside the cell promote cancer cell invasion. On the other hand, these same changes sensitize cancer cells to LMP. As an example, increased size of the lysosomes can make the lysosomes more fragile and more prone to leakage (Kallunki et al., 2013).

Although the mechanisms underlying GBM cell sensitivity to LMP were not specifically addressed in this study, it is important to discuss. Based on the literature, the mechanism of how CADs induce LMP first involves accumulation of CADs into the lysosomes and subsequently detachment of acid sphingomyelinase from their membranes, which alters the lipid composition decreasing its stability. Acid sphingomyelinase normally catalyzes hydrolysis of sphingomyelin into ceramide and phosphorylcholine, but increased levels of acid sphingomyelinase within the lysosomes reduces lysosomal membrane integrity and induces cathepsin release via LMP (Kornhuber et al., 2010; Serrano-Puebla and Boya, 2018). Furthermore, in a previous study, reduced acid sphingomyelinase enzyme activity was associated with increased lysosomal volume and decreased stability, which increased sensitivity of cells to LMP (Petersen et al., 2010) potentially explaining the increased sensitivity of cancer cells to treatments with CADs.

Potential of the lysosome-targeted therapies have been investigated in preclinical studies including several types of cancers. Treatment of tumor cells with a lysosomotrophic agent siramesine induced cell death via LMP. Furthermore, treatment of breast and fibrosarcoma xenografts with siramesine significantly suppressed tumor growth in vivo (Ostenfeld et al., 2005). Lysosomal disruption by mefloquine, an antimalarial agent, selectively targeted leukemic cells and demonstrated therapeutic efficacy in subcutaneous xenografts of human and murine acute myeloid leukemia by significantly decreasing the tumor growth (Sukhai et al., 2013). To conclude, alternative cell death pathways capable of causing cytotoxicity in therapy-resistant cancer cells, including GBM, have gained an increased interest as means to target these cells by LMP-inducing agents. Further studies are needed to evaluate their possible clinical potential. We aim to test clemastine in a clinical trial of patients with GBM and a preliminary discussion about the possibility has been initiated with oncologists working in Finland.

4. CD109-GP130 interaction drives glioblastoma stem cell plasticity and chemoresistance through activation of STAT3 (II)

GSC plasticity has recently emerged as an important mediator of intratumoral heterogeneity and therapy resistance in GBM. GSCs are considered as attractive therapeutic targets. However, despite significant efforts, direct therapeutic targeting of GSCs has been challenging whereas targeting the stem cell niche or the GSC plasticity are promising strategies to eradicate the GSCs. In the study II, we investigated the molecular mechanisms underlying GSC plasticity and therapy resistance. We discovered that CD109 physically interacts with glycoprotein 130 (GP130) to regulate activation of the STAT3 pathway. We further demonstrate the importance of the CD109/STAT3 axis in GSCs plasticity and tumorigenicity. Finally, targeting CD109/STAT3 axis sensitized the GSCs to TMZ chemotherapy suggesting that targeting CD109/STAT3 axis has potential to overcome therapy resistance in GBM.

4.1. CD109/STAT3 axis drives the stemness of glioblastoma cells

We investigated the expression of CD109 in our GSCs and detected high expression together with the GSC markers SOX2 and Olig2 (Suva et al., 2014). When the GSC cultures were exposed to serum, the gliospheres rapidly differentiated into adherent cultures. The serum-induced cell differentiation caused a rapid downregulation of the expression of CD109 and the GSC markers SOX2 and Olig2 supporting an association between CD109 expression and maintenance of GSC stemness properties. Moreover, the GSCs expressed constitutively phosphorylated STAT3, which has been previously implicated in the maintenance of GSC stemness (Sherry et al., 2009; Wang et al., 2009).

To investigate the functional role of CD109 on GSC stemness, we silenced CD109 expression by using lentiviral-mediated shRNAs in BT12 and BT13 cells. Silencing efficiencies of the two CD109-targeting shRNAs were confirmed at mRNA and protein levels. The shCD109#1 showed efficient silencing of CD109 expression whereas silencing with the shCD109#2 was partial. To study the association between CD109 and stemness, we first analyzed the expression of several GSC markers in response to CD109 silencing. CD109 silencing decreased SOX2 and Olig2 expression at protein level. Consistent with the decreased protein levels, mRNA level expression of several GSC markers was downregulated in CD109-silenced GSCs compared to the non-targeted controls. Overall, the BT13 cells showed a more profound effect compared to the BT12 cells. Next, we studied the effect of CD109 silencing on other functional properties of GSCs. CD109 silencing significantly decreased cell proliferation and colony formation from single cells in a semi-solid methylcellulose matrix in vitro compared to the non-targeted controls suggesting reduced self-renewal capacity. Moreover, CD109-silenced GSCs demonstrated significantly inhibited growth properties in a 3D fibrin matrix that better models the mechanical properties of the brain tissue. To further investigate the association of CD109 with pSTAT3, we analyzed the pSTAT3 levels in CD109-silenced GSCs and non-targeted control cells. CD109 silencing markedly reduced the pSTAT3 levels in several GSC cultures independent of the GBM subtype. Taken together, these results verify the association between CD109 and pSTAT3 that was observed in the clinical GBM samples and suggests that the CD109/STAT3 axis maintains the stemness properties of GSCs.

4.2. CD109-GP130 interaction regulates the activation of the IL-6/STAT3 pathway

Since the CD109/STAT3 axis had not been previously reported in GBM, we decided to further investigate the association between CD109 and STAT3. STAT3 signaling plays a very central role in cancer as it has been shown to promote cell proliferation, cell survival, invasion, angiogenesis, and stemness (Yu et al., 2014). IL-6/GP130 signaling is the central signaling pathway that activates STAT3 (Jones et al., 2011). In the classic IL-6 signaling pathway, IL-6 binds to the plasma membrane-bound IL-6 receptor-α (IL-6R), which forms a heteromeric complex with the common co-receptor and signal transducer GP130. Formation of the signaling complex activates

the intracellular signaling via the JAK/STAT3 signaling pathway (Figure 9) (Johnson et al., 2018). Whereas GP130 is widely expressed in most cells in the human body, the expression of IL-6R is restricted to only a few cell types such as hepatocytes and leukocytes (Jones et al., 2011). However, GSCs have been previously reported to express IL-6R and stimulation of GSCs with IL-6 increased the levels of phosphorylated STAT3 (Wang et al., 2009).

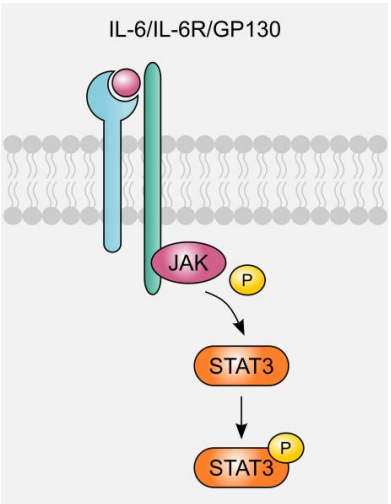

Figure 9. IL-6/GP130/STAT3 signaling pathway. IL-6 binds to the IL-6 receptor (IL6R) which then forms a complex with GP130 and activates signal transduction via the JAK/STAT3 pathway. Modified from Johnson et al., 2018.

We detected that the non-targeted control GSCs responded to IL-6 stimulation by phosphorylating STAT3 whereas the CD109-silenced GSCs did not. Lack of pSTAT3 activation in CD109-silenced GSCs was likely due to decreased levels of the GP130 co-receptor. To further establish the relationship between CD109 and GP130 and to gain mechanistic insight into the STAT3 signaling pathway, we hypothesized that these proteins, both expressed at the plasma membrane, could physically interact. Based on amino acid sequence analysis, CD109 has a thioester site which has been previously suggested to mediate covalent binding (Lin et al., 2002). Co-immunoprecipitation assay supported a physical interaction between CD109 and GP130. To verify the result, we utilized PLA assay which allows direct detection of protein-protein interactions in situ. Interestingly, the PLA assay confirmed the physical interaction between CD109 and GP130. Our further analyses demonstrated that the physical interaction was specifically mediated by the 180-kDa subunit of CD109 and not by the 25-kDa subunit (Figure 10A and B). Thus, physical interaction between the 180-kDa CD109 and GP130 promotes activation of the STAT3 pathway which maintains the stemness of GSCs.

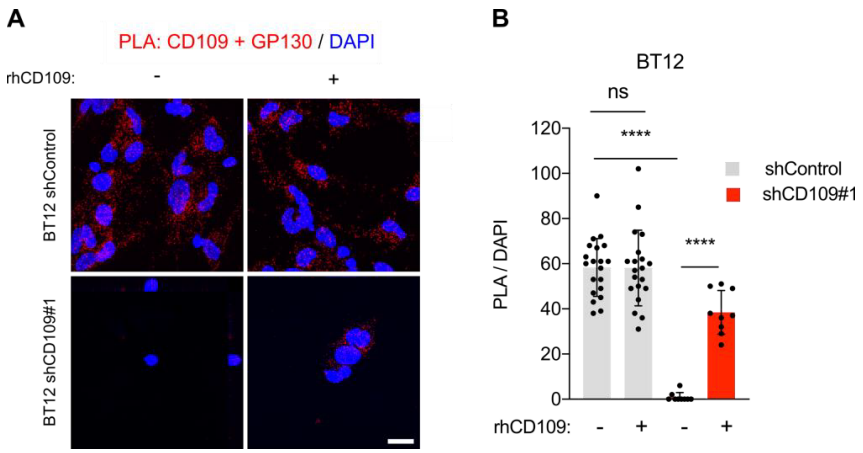

Figure 10. CD109 physically interacts with GP130. A) Representative images of the PLA experiment. PLA signal is shown in red. Nuclei were counterstained with DAPI (blue). Scale bar: 20 µm. B) Quantification of PLA signal per cell. The figures have been modified from Filppu et al., 2021 with the permission from the American Society for Clinical Investigation.

4.3. Induced astrocytic-like state sensitizes the cells to chemotherapy

A key functional property of GSCs is the ability to differentiate into multiple lineages (Lee et al., 2006). Since CD109-silenced GSCs demonstrated significantly impaired stemness properties, we investigated whether the cells would differentiate. A significant increase in the mRNA level expression of glial fibrillary acidic protein (*GFAP*), a marker for mature astrocytes, was detected by qRT-PCR analysis in response to CD109 silencing suggesting that GSCs would differentiate towards astrocytic lineage. A recent study showed that *GFAP* expression was enriched in the astrocytic-like (AC-like) cell state that corresponds to the CL subtype of GBM (Neftel et al., 2019). To get a more comprehensive picture of the gene expression alterations, we performed bulk RNA-sequencing analysis of CD109-silenced GSCs and their non-targeted control cells. Data analysis demonstrated consistent upregulation of the expression of AC-like state genes in response to CD109 silencing. We validated increased expression of selected CL subtype genes using qRT-PCR. Thus, our results demonstrate that CD109 is essential for the maintenance of GSC stemness and plasticity and its silencing induces a phenotypic shift towards the AC-like state.

STAT3 plays a central role in promoting cancer cell survival (Yu et al., 2014). Accordingly, we detected partial apoptosis in response to CD109 silencing in GSCs that was likely mediated by inhibition of STAT3 activity. Especially the proneural ZH305 cells were highly sensitive to CD109 silencing. Our results are consistent with the previous studies that demonstrated both partial cell differentiation and apoptosis in GSCs in response to STAT3 inhibition with small molecule inhibitors or genetic targeting of IL-6R (Sherry et al., 2009; Wang et al., 2009).

Previous studies have demonstrated that GSCs are highly resistant to radiation and chemotherapy (Bao et al., 2006; Liu et al., 2006). GSC plasticity has been recently suggested as an important mediator of therapy resistance since it allows phenotypic adaptation by dynamic state transitions in response to therapy (Prager et al., 2020). To test whether CD109 silencing would sensitize the GSCs to TMZ chemotherapy we treated control and CD109-silenced GSCs with varying concentrations of TMZ. Interestingly, CD109-silenced GSCs were significantly more sensitive to treatment with TMZ during a four-day treatment period in vitro compared to non-targeted control cells. Moreover, combined treatment with STAT3 inhibitor (Stattic) and TMZ similarly sensitized the wild type GSCs to chemotherapy. CD109 has been recently linked to enhanced radiotherapy resistance in GBM (Minata et al., 2019), but studies addressing chemoresistance were lacking. Our results support an important role for CD109 in mediating chemoresistance and suggest that targeting CD109/STAT3 axis sensitizes the GSCs to chemotherapy.

4.4. CD109 is crucial for glioblastoma growth in vivo

To investigate the role of CD109 in tumor formation in vivo, we established intracranial orthotopic xenografts derived from the patient-derived GSCs enriched with MES and PN signatures. As described above, the cells were highly tumorigenic upon xenotransplantation with distinctive characteristic growth patterns between the GBM subtypes. CD109 silencing completely inhibited tumorigenesis of the PN BT18 and ZH305 cells that grow very diffusively, as well as the growth of MES BT13 which forms large and highly angiogenic bulky tumor masses. In contrast, the tumor initiation was not completely inhibited by CD109 silencing in the MES BT12 model, which displays a multimodal growth pattern including formation of large primary tumor, smaller satellite tumors, and collective and single cell invasion (Figure 11A and B).

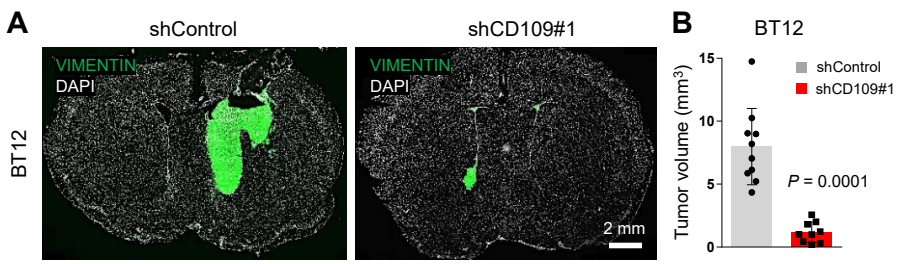

Figure 11. CD109 silencing markedly impaired GBM growth in vivo. A) Representative images of BT12 xenografts. Tumor cells were visualized with vimentin (green) and nuclei were counterstained with DAPI (white). Scale bar: 2 mm. B) Quantification of the tumor volume of BT12 control and CD109-silenced xenografts. The figures have been modified from Filppu et al., 2021 with the permission from the American Society for Clinical Investigation.

Further characterization of the control and the small tumors derived from the CD109-silenced MES BT12 model revealed decreased levels of pSTAT3 and expression of the stem cell markers SOX2 and Olig2 in the CD109-silenced samples compared to the controls as demonstrated by Western blot analyses of the whole tumor extracts. Possible reasons why the tumor growth in the MES BT12 model was not completely inhibited after CD109 silencing could be its highly multimodal growth pattern in vivo that might increase its resistance to CD109 silencing. As noted above, the loss of stemness phenotype was more profound in the BT13 cells compared to the BT12 cells and therefore the BT12 cells might have intrinsic resistance to CD109 silencing possibly due to higher degree of cellular heterogeneity or increased adaptation potential.

Beyond the tumor cells, we observed significant alterations in the vasculature of the CD109-silenced MES BT12 xenografts. Analysis of the tumor blood vessels revealed a significant reduction in both size and density of the blood vessels in the CD109-silenced xenografts compared to the controls. Furthermore, CD109 silencing in GSCs in vitro increased secretion of pro-inflammatory cytokines (Fas ligand, IL-1β, and TNF-α) to the cell culture medium and increased the mRNA level expression of angiopoietin 2 (*ANGPT2*). Angiopoietin 2 has a well-established role as a promoter of endothelial destabilization, permeability, and pericyte deficiency (Armulik et al., 2011). Immunostainings of the xenograft tumors with pericyte markers α-smooth muscle actin together with NG2 or PDGFRβ and NG2 demonstrated lack of pericyte coverage in the vasculature of CD109-silenced xenografts.

OPC-like cells are characterized by PDGFRα and NG2 expression and these cells are the only resident CNS cell population that express NG2 (Dougherty et al., 2012; Nayak et al., 2018). We identified PDGFRα and NG2 positive glioma-associated OPC-like cells adjacent to the tumor endothelium in the xenografts derived from non-targeted control cells consistent with observations by Huang and colleagues (Huang et al., 2014). However, in the CD109-silenced xenografts the number of OPC-like cells was significantly reduced suggesting a loss of these progenitor cells in the GBM perivascular niche. Interestingly, Huang et al. also demonstrated that OPC-like cells promoted tumor vascular remodeling and angiogenesis suggesting that the OPC-like cells contribute to tumor progression (Huang et al., 2014). OPC-like cells have been suggested as the cell of origin in a mouse model of glioma, although their contribution to GBM is still relatively poorly characterized (Liu et al., 2011). Another highly interesting feature of the OPC-like cells is that they have been demonstrated to receive direct synaptic input from neurons (Nayak et al., 2018). To summarize, these results support an important role for CD109 in promoting tumor initiation and progression of GBM (Figure 12).

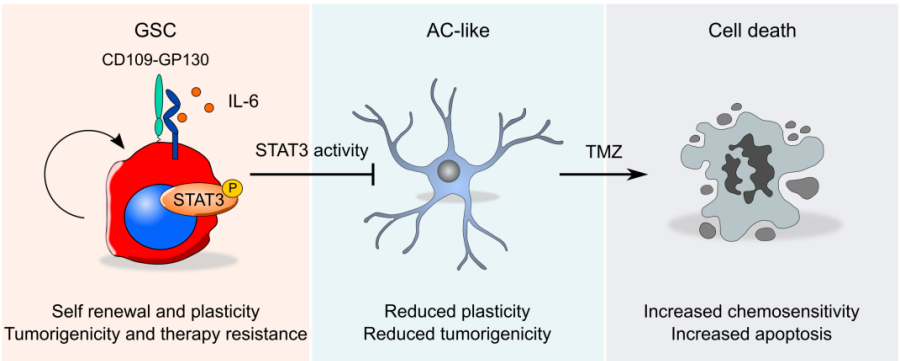

Figure 12. Schematic illustration of the CD109-mediated plasticity and tumorigenicity in GSCs. CD109 physically interacts with GP130 to promote activation of the IL-6/STAT3 signaling pathway which maintains GSC stemness, plasticity and tumorigenicity. Inhibition of STAT3 activity in GSCs induced astrocytic-like cell differentiation, impaired tumorigenicity, and sensitized the cells to TMZ.

4.5. Potential of targeting CD109/STAT3 axis in glioblastoma

The results of our study indicate that targeting the CD109/STAT3 axis in GBM could have therapeutic potential to inhibit tumor growth and sensitize the tumor cells to chemotherapy. However, strategies to directly target CD109 are currently unavailable. The IL-6/STAT3 pathway has been a target for development of several drugs. Current status of clinical trials targeting the IL-6/STAT3 signaling in cancer using anti-IL-6 or anti-IL-6R antibodies or direct STAT3 inhibitors has been extensively reviewed recently (Johnson et al., 2018). Several agents targeting IL-6, IL-6R, or JAKs have been approved by the FDA for treatment of certain inflammatory conditions such as rheumatoid arthritis and testing these agents in preclinical models of solid tumors have demonstrated antitumor efficacy (Johnson et al., 2018). For example, an antibody targeting IL-6, siltuximab, demonstrated antitumor efficacy in ovarian (Coward et al., 2011) and lung cancers (Song et al., 2014). Despite the results obtained in preclinical studies, neither of these agents have demonstrated efficacy against solid tumors in clinical trials so far (Johnson et al., 2018).

Due to the central role of STAT3 in promoting tumor cell growth and survival (Yu et al., 2014), there has been a significant effort to develop direct STAT3 inhibitors. However, this task has proven difficult, since STAT3 is an intracellular transcription factor that lacks enzymatic activity. In addition, the BBB present in the brain tumor microenvironment efficiently hampers delivery of potential therapeutic agents to the tumor. In a preclinical study of colorectal cancer, STAT3 inhibition by Stattic sensitized the tumor cells to radio- and chemotherapy in vitro and significantly inhibited tumor growth of subcutaneous xenografts in vivo (Spitzner et al., 2014). Inhibition of STAT3 activity by a small molecule JAK-1/2 inhibitor momelotinib sensitized the long-term serum cultured U251MG GBM cells to TMZ in vitro and combined treatment with momelotinib and TMZ demonstrated significant growth inhibition of orthotopic xenografts in vivo and enhanced tumor cell apoptosis (Liu et al., 2019). Several phase I/II clinical trials investigating the potential of STAT3 inhibitors on advanced-stage cancers are currently ongoing (Johnson et al., 2018).

IL-6/STAT3 pathway is also an interesting therapeutic target because STAT3 induces the expression of immunosuppressive factors in the tumor microenvironment. Therefore, targeting the IL-6/STAT3 pathway could potentially stimulate antitumor immunity (Johnson et al., 2018). In a preclinical study investigating colorectal cancer, STAT3 inhibition by nifuroxazide impaired metastasis formation and increased antitumor immunity by reducing the number of myeloid-derived suppressor cells and increasing the infiltration of CD8+ T-cells in the tumors (Ye et al., 2017). Another study investigating the potential of a BBB permeable small molecule STAT3 inhibitor WP1066 demonstrated that STAT3 inhibition enhanced immune responses in patients with malignant glioma by upregulating costimulatory molecules in microglia/macrophages and stimulating effector T cell function (Hussain et al., 2007).

Even though STAT3 is an interesting and promising therapeutic target, there are several concerns regarding its inhibition in treatment of cancer. Although STAT3 is often hyperactivated in cancer, it is essential for normal cell physiology and during development. It has been previously demonstrated that knockdown of *STAT3* leads to embryonic lethality (Jones et al., 2011) demonstrating the essential role for STAT3 in normal development. STAT3 is also crucial for the maintenance of self-renewal and pluripotency of embryonic stem cells (Niwa et al., 1998; Chen et al., 2015). Furthermore, activation of the STAT3 signaling in the developing nervous system promotes differentiation of neural stem/progenitor cells into astrocytes (Bonni et al., 1997; Nakanishi et al., 2007).

Based on our results, targeting CD109/STAT3 axis could reprogram GSCs into a less tumorigenic and drug-sensitive state via induction of astrocytic-like cell differentiation. An interesting idea to potentially target GSCs is to induce their differentiation to inhibit tumorigenicity and reduce therapy resistance. However, a study investigating the therapeutic potential of GSC differentiation towards mature astrocytes reported an important finding that GSCs failed to undergo terminal cell differentiation to a post-mitotic state (Caren et al., 2015) suggesting that differentiation therapy alone may not be effective in treatment of GBM. Another study showed that a population of GSCs expressing Achaete-scute homolog 1 (ASCL1) differentiated towards neuronal lineage in response to inhibition of Notch signaling which also reduced tumorigenicity of the cells (Park et al., 2017). It should be noted that neuronal signature is commonly upregulated in the PN subtype gliomas and therefore neuronal differentiation may not represent universal strategy across gliomas, but rather in a subset of GBM patients.

One possible approach to target GSC plasticity is an induction of a cell state that is less tumorigenic, and more responsive to therapies. As suggested recently, induction of the AC-like cell state could potentially be therapeutically interesting. The AC-like cell state is common across gliomas and appears to be less proliferative and less tumorigenic, although more studies may be needed to warrant this strategy (Suva and Tirosh, 2020). To summarize, targeted eradication of GSCs represent a clinically relevant strategy to improve patient survival. However, development of such therapies has been difficult. Our results suggest that a combinatorial approach of induction of the AC-like cell state and TMZ chemotherapy could potentially reduce tumorigenicity and overcome therapy resistance of GSCs.

CONCLUSIONS AND FUTURE PROSPECTS

GBM is one of the most difficult cancers to treat with dismal prognosis. Despite significant research efforts, no pharmacological intervention that would increase the overall survival of patients exist. Most targeted therapies against GBM have failed to demonstrate improved overall survival. While immunotherapy has been a promising treatment option in several solid tumors, clinical trials have not demonstrated efficacy for immunotherapy in GBM (McGranahan et al., 2019). Together these challenges highlight the complexity of this disease and the need for development of more efficient therapeutic strategies. In this study, we investigated the molecular mechanisms underlying GBM progression and therapy resistance. Identification of specific vulnerabilities could lead to development of more efficient therapeutic strategies.

In this study, we first showed that the invasive GBM cells were unexpectedly vulnerable to inhibition of their lysosome membrane integrity. Both silencing of MDGI expression and treatment of GBM cells with clemastine resulted in lysosome-mediated cell death. In our preclinical study, clemastine eradicated the highly invasive tumor cells in vivo suggesting that clemastine could be repurposed for treatment of GBM. We aim to test clemastine in a clinical trial of patients with GBM in the near future. A preliminary discussion about the possibility of a clinical trial has been initiated with oncologists working in Finland.

Previous studies have suggested repurposing of antihistamines and specifically CADs for treatment of cancer (Ellegaard et al., 2016; Faustino-Rocha et al., 2017) thus supporting our findings. Since it was suggested by the previous studies that CADs could revert multidrug resistance and sensitize cancer cells to chemotherapy (Jaffrezou et al., 1995; Drinberg et al., 2014; Ellegaard et al., 2016; An et al., 2017), it would be interesting to study clemastine treatment in combination with TMZ chemotherapy in a preclinical setting to test whether the combination treatment would target both invasive cells and inhibit the growth of the tumor mass. Although clemastine is safe as a monotherapy (Turski et al., 2018), there are currently no reports available about combination of clemastine and TMZ chemotherapy. For the safety concerns, it would be important to study the combination treatment for possible side effects in a preclinical study before a clinical trial. Furthermore, the effect of clemastine on the BBB permeability could be studied. Tesmilifene, a drug with antihistamine action, increased the BBB permeability in a rat glioma model (Walter et al., 2015). Increased BBB permeability could enhance the delivery of chemotherapies to the tumor and improve anti-tumor efficacy.

In this study, we also demonstrated that physical interaction between CD109 and GP130 activates the STAT3 signaling pathway in GSCs. We further established the significance of CD109/STAT3 axis in the maintenance of stemness, plasticity, tumorigenicity, and chemoresistance of GSCs. Development of STAT3 inhibitors has been challenging and as an intracellular transcription factor STAT3 has been considered as an undruggable target (Johnson et al., 2018). Moreover, there are several concerns regarding STAT3 inhibition. STAT3 is essential for normal cell physiology and development as it promotes self-renewal and maintains pluripotency of embryonic stem cells (Niwa et al., 1998; Chen et al., 2015) as well as promotes differentiation of

neural stem/progenitor cells into astrocytes in the developing nervous system (Bonni et al., 1997; Nakanishi et al., 2007). Considering the concerns and difficulties regarding STAT3 inhibition, targeting the CD109/STAT3 axis at the level of CD109 represents a promising idea. However, specific inhibitors against CD109 are currently unavailable. Our future studies will concentrate on characterizing different approaches to specifically target CD109. A collaboration to develop an antibody-based method to inhibit CD109 has been recently established.

Our results suggest that targeting the CD109/STAT3 axis could reprogram GSCs into a less tumorigenic and drug-sensitive state. Increasing evidence from the recent single-cell RNA-sequencing studies show that GSCs seem not to be a functionally defined population but rather cell states that are highly plastic and dynamically transfer from one state to another thus generating intratumoral heterogeneity which makes their therapeutic targeting very challenging (Dirkse et al., 2019; Neftel et al., 2019). We demonstrated that silencing of CD109 expression induced cell differentiation and a shift towards the AC-like cell state. The AC-like cells were less proliferative, less tumorigenic, and more sensitive to chemotherapy treatment. Our results suggest a combinatorial approach of induction of AC-like cell state and TMZ chemotherapy as a potential strategy to target the GSCs and to overcome their therapy resistance. Importantly, another recent study shows that AC-like cells appear to be less tumorigenic and inducing the AC-like state in GBM could represent a potential strategy to target GSCs plasticity, thus supporting our findings (Suva and Tirosh, 2020).

Finally, studies about CD109 have been mostly focusing on cancer, although CD109 is also expressed by some normal cell types including for example activated T-cells and endothelial cells. The function of CD109 in normal cell types remains largely unexplored. CD109 expression by activated T-cells suggest a function for CD109 in the immune system or regulation of the immune response, but this remains to be explored. A recent study investigating tumor-associated endothelium in vivo, suggested that CD109 would promote blood vessel stability (Yamakawa et al., 2018). Our unpublished findings (Filppu et al., unpublished results) encourage to further investigate the role of CD109 in the TME and more specifically interactions and cellular crosstalk between tumor cells and stromal cells such as immune cells and endothelial cells. This thesis provides important insight into the molecular mechanisms underlying GBM progression and therapy resistance.

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Pauliina Filppu

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