Aus der Klinik für Neurochirurgie

Geschäftsführender Direktor: Prof. Dr. med. Christopher Nimsky

Des Fachbereichs Medizin der Philipps-Universität Marburg

Identification of microRNA markers for glioblastoma progression

Inaugural-Dissertation zur Erlangung des Doktorgrades der gesamten Humanmedizin (Dr. med.)
dem Fachbereich Medizin der Philipps-Universität Marburg

vorgelegt von

Lara Evers
aus Buchholz in der Nordheide
Marburg, 2023

Angenommen vom Fachbereich Medizin der Philipps-Universität Marburg am:
05.12.2023

Gedruckt mit Genehmigung des Fachbereichs Medizin

Dekanin: Prof. Dr. Denise Hilfiker-Kleiner
Referent: Prof. Dr. Jörg-Walter Bartsch
Korreferent: Prof. Dr. Malte Buchholz

Diese kumulative Promotion umfasst eine Zusammenfassung der beiden folgenden Publikationen:

1. The Metalloprotease-Disintegrin ADAM8 alters the tumor suppressor miR-181a-5p expression profile in glioblastoma thereby contributing to its aggressiveness

Agnes Schäfer *, Lara Evers *, Lara Meier, Uwe Schlomann, Miriam H. A. Bopp, Gian-Luca Dreizner, Olivia Lassmann, Aaron Ben Bacha, Andreea-Cristina Benescu, Mirza Pojskic, Christian Preußer, Elke Pogge von Strandmann, Barbara Carl, Christopher Nimsky and Jörg W. Bartsch
*These authors have contributed equally to this work

2022 Frontiers in Oncology, DOI: 10.3389/fonc.2022.826273

2. Identification of Dysregulated microRNAs in Glioblastoma Stem-Like Cells

Lara Evers *, Agnes Schäfer *, Raffaella Pini, Kai Zhao, Susanne Stei, Christopher Nimsky and Jörg W. Bartsch
* These authors have contributed equally to this work

2023 Brain Sciences, DOI: 10.3390/brainsci13020350

Table of content

I. List of abbreviations

ADAM8	A disintegrin and metalloproteinase domain-containing protein 8
AKT	Protein kinase B
ATM	Ataxia-teleangiectasia mutated
Bcl-2	B-cell lymphoma 2
bFGF	bovine fibroblast growth factor
Cas9	CRISPR-associated protein 9
CCL13	Chemokine (C-C motif) ligand 13
CD	Cluster of differentiation
CREB	Cyclic adenosine monophosphate-responsive element binding protein
CRISPR	Clustered regularly interspaced short palindromic repeats
CT	Computer tomography
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ERK	Mitogen-activated protein kinase
EV	Extracellular vesicle
FAK	Focal adhesion kinase
FCS	Fetal calf serum
GBM	Glioblastoma
GFAP	Glial fibrillary acidic protein
GSC	Glioblastoma stem-like cell
HIF	Hypoxia-inducible factor
IDH	Isocitrate dehydrogenase
IGF1R	Insulin-like growth factor 1 receptor
ITGB1	Integrin beta-1
JAK2	Janus kinase 2
KEGG	Kyoto Encyclopedia of Genes and Genomes
Let-7	Lethal-7
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MGMT	O-6-methylguanine-DNA methyltransferase
miRNA	microRNA
MMP	Matrix metalloproteinase
MR	Magnetic resonance
mRNA	messenger RNA

MSI2	Musashi-2
NK	Natural killer
NSCLC	Non-small-cell lung cancer
onco-miRNA	Oncogene miRNA
OPN	Osteopontin
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide 3-kinase
Pri-miRNA	Primary miRNA
PTEN	Phosphatase and tensin homolog
qPCR	Quantitive polymerase chain reaction
RAS	Rat sarcoma virus
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
SOX2	Sex determining region Y-box 2
STAT3	Signal transducer and activator of transcription 3
TAM	Tumor-associated macrophage
TERT	Telomerase reverse transcriptase
TME	Tumor microenvironment
TMZ	Temozolomide
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

II. List of figures

1. Introduction

1.1 Glioblastoma

Glioblastoma (GBM) is the most prevalent and lethal primary brain malignancy in adults (Ostrom et al. 2020). This high-grade brain tumor mainly manifests in older adults, as the average age of onset is 65 years (Ostrom et al. 2020). Epidemiologically, the incidence of this brain tumor ranges from 4-6 cases per 100.000 people, and men are more likely to suffer from GBM (Philips et al. 2018). However, the cause of this currently incurable disease remains elusive. A few known risk factors include exposure to ionizing radiation and genetic disorders, such as Li-Fraumeni syndrome and neurofibromatosis (Tan et al. 2020). Initially, GBM patients often present with unspecific symptoms dependent on tumor localization, like headaches, nausea, seizures, focal deficits, and changes to personality, memory, and vigilance (McKinnon et al. 2021). To make a diagnosis, computer tomography (CT) imaging and, more prominently, magnetic resonance (MR) imaging are often combined with definitive pathological confirmation (Silantyev et al. 2019). To add to the complexity, the composure of GBM is highly heterogeneous (Soeda et al. 2015). For instance, different tumor zones show distinctive metabolic activities, as MR-spectroscopy imaging suggests (Bulik et al. 2013). Pathologically, primary brain tumors are characterized by the World Health Organization (WHO). 2021 adapted, the WHO classification categorizes GBM as WHO IV, the most aggressive primary brain malignancy (Wen and Packer 2021). At the molecular level, GBM is characterized by numerous genetic alterations including but not limited to an isocitrate dehydrogenase (IDH) wild-type status, an amplified or mutated epidermal growth factor receptor (EGFR), loss of p53 function, and telomerase reverse transcriptase (TERT) promoter mutations (Gritsch et al. 2022). The only broadly established prognostic histopathological biomarker is the methylation status of the O-6-methylguanine-DNA methyltransferase (MGMT) promoter region (Weller et al. 2009; Butler et al. 2020). A methylated MGMT promoter is associated with prolonged survival due to a favorable response to the alkylating chemotherapeutic agent Temozolomide (TMZ), the gold standard in GBM chemotherapy (Stupp et al. 2005).

Even though GBM patients are aggressively treated by maximum safe surgical resection, adjuvant radiation, and chemotherapy, most frequently using TMZ, GBM is a highly recurrent and progressive disease (Anjum et al. 2017). Recurrent tumors are often resistant to the available treatment options. As a result, the median survival of 12 up to 15 months remains poor (Stupp et al. 2009). Therefore, identifying and testing new therapeutic agents is a crucial current research task. So far, recent treatment attempts like the vascular endothelial growth factor (VEGF) inhibitor Bevacizumab could not significantly prolong the overall survival of GBM patients (Chinot et al. 2014).

At the molecular level, dysregulated vital signaling pathways have been identified to contribute to GBM aggressiveness and progression. For instance, overstimulation of the mitogen-activated protein kinase (MAPK) signaling cascade promotes cell migration and invasion by inducing the transcription of oncogenes like matrix metalloproteinases (MMPs, Lakka et al. 2002). Second, the signal transducer and activator of transcription 3 (STAT3) pathway promotes tumor growth and angiogenesis (Priester et al. 2013; Li et al. 2021). Due to the regulation of the cell cycle, apoptosis, and cancer stem cell maintenance, the phosphoinositide 3-kinase (PI3K)/ protein kinase B (AKT) pathway plays a central role in GBM carcinogenesis (Barzegar Behrooz et al. 2022). Nonetheless, many regulatory mechanisms of the complex GBM biology remain elusive.

1.1.1 Glioblastoma stem-like cells

A small but highly influential group of GBM tumor cells, named glioblastoma stem-like cells (GSCs), shows distinctive characteristics compared to the bulk tumor mass, such as capacities for multi-lineage differentiation and self-renewal (Lathia et al. 2015). With these properties comparable to those of neural progenitor cells and pluripotent stem cells, GSCs are currently viewed as the origin of the almost inevitable tumor recurrence (Chen et al. 2012; Gimple et al. 2019). Expression of stemness markers such as the transmembrane glycoproteins cluster of differentiation (CD) 133 and CD44 or the transcription factor sex determining region Y-box 2 (SOX2) distinguish GSCs from the differentiated bulk tumor mass at the molecular level (Aghajani et al. 2019; Gimple et al. 2019). On the other hand, elevated glial fibrillary acidic protein (GFAP) levels are commonly used to characterize differentiated astrocytic tumor cells (Ricci-Vitiani et al. 2008). Nevertheless, proper GSC characterization remains challenging due to the astonishing phenotypic plasticity of GBM (Soeda et al. 2015).

Maintenance of stem cell properties is a complex interaction of GSCs and the surrounding tumor microenvironment (TME). For instance, there is strong evidence that hypoxia stabilizes the GSC phenotype (Soeda et al. 2009). Thus, GSCs are frequently located apart from the bulk tumor mass in perivascular and hypoxic niches (Garnier et al. 2019). As a result, GSCs are insufficiently targeted by surgical tumor resection (Lathia et al. 2015). Finally, GSCs also contribute to GBM progression by enhancing radiation- and chemotherapy resistance, resulting in rapid, hardly treatable relapses (Bao et al. 2006; Chen et al. 2012).

1.1.2 Glioblastoma tumor microenvironment

The TME is implicated in tumor cell invasion, neovascularization, and facilitation of the extraordinary intratumoral plasticity of GBM (Dapash et al. 2021). Therefore, the TME

comprises a complex interactive network of invading tumor cells, GSCs, immune cells, resident glial and neural cells, and fibroblastic cells (Codrici et al. 2022). Tumor cells, dominantly GSCs, form the TME into perivascular and hypoxic niches associated with genetic instability and cancer progression (Folkins et al. 2009; Garnier et al. 2019). Another principal feature of the TME is its highly immunosuppressive nature, even though recruited immune cells represent one of the most predominant cell types within the TME (Pires-Afonso et al. 2020). For instance, tumor-associated macrophages (TAMs) are often polarized into an anti-inflammatory and, thus, tumor-supportive M2-phenotype (Wu et al. 2010; Gjorgjevski et al. 2019). In addition, invasion is closely associated with extracellular matrix (ECM) remodeling (Vollmann-Zwerenz et al. 2020). In this context, zinc-dependent endopeptidases, prominently MMP9, degrade matrix components, thereby augmenting GBM aggressiveness (Li et al. 2016). The tumor cross-talk mediated by direct cell-cell interactions, secretory molecules, and extracellular vesicles (EVs) is fundamental to the TME hallmarks (Cavallari et al. 2020). All cell types within the TME communicate by encapsulating EVs, and their heterogeneous cargo contains lipids, proteins, and nucleic acids (van Niel et al. 2018). Because these nano-molecules can pass the blood-brain barrier and circulate in body fluids, including cerebrospinal liquor and the bloodstream, EVs are also intensely studied as future biomarkers and therapeutic vehicles (Nazimek and Bryniarski 2020).

1.2 micro-RNAs

Among regulatory, non-coding ribonucleic acids (RNAs), microRNAs (miRNAs) play a pivotal role in regulating gene expression at the posttranscriptional level (Fabian et al. 2010). These single-stranded, tiny molecules of approximately 21 to 23 nucleotides bind by complementary base-pairing to their target messenger RNAs (mRNAs), thereby forming an RNA-induced silencing complex (RISC) and inhibiting protein translation or even inducing mRNA degradation (Green et al. 2016). Biogenesis of these regulatory molecules is a complex multi-step process, including the transcription of a hairpin-loop primary miRNA (pri-miRNA) and intranuclear and cytoplasmatic processing to a single-stranded mature miRNA (O'Brien et al. 2018).

Not only do miRNAs silence gene expression in their cells of origin, but they are also secreted into body fluids as circulating miRNAs (Turchinovich et al. 2011). These nano-molecules shape the TME, especially by influencing the immune response to invading cancer cells (Buruiană et al. 2020). To add to the complexity, investigations suggest that specific miRNAs function as tumor suppressor miRNAs or oncogene miRNAs (onco-miRNAs) dependent on the type of cancer (Ali Syeda et al. 2020). In the case of GBM, dysregulated miRNAs are associated with critical hallmarks of GBM progression, for

instance, tumor cell invasion, immune evasion, and induction of angiogenesis (Møller et al. 2013; Shea et al. 2016). Thus, regulatory pathways of miRNA expression are an important object of research.

1.2.1 miR-181a-5p

In different cancer types, members of the miR-181 family have diverse functions as either onco-miRNAs or tumor suppressors (Indrieri et al. 2020). In glioblastoma, however, miR-181a-5p is a known tumor suppressor, for instance, by enhancing radiation- and chemotherapy sensitivity (Chen et al. 2010; Wen et al. 2020). Generally, miR-181a-5p is scarcely expressed in GBM, as miR-181a-5p levels are inversely correlated with glioma grade (Shi et al. 2008). Moreover, low miR-181a-5p levels are correlated with reduced overall survival and poor functional patient status (Valiulyte et al. 2022). At the molecular level, miR-181a-5p targets Osteopontin *(*OPN) mRNA, thereby silencing OPN-mediated angiogenesis (Marisetty et al. 2020; Li et al. 2021). Furthermore, the sialoprotein OPN is an essential mediator of the TME by attracting TAMs, suggesting a regulatory role of miR-181a-5p in tumor immune modulation (Wei et al. 2019; Marisetty et al. 2020). In addition, miR-181a-5p targets the anti-apoptotic oncogene B-cell lymphoma 2 (Bcl-2), thereby promoting apoptosis when highly abundant (Chen et al. 2010). Interestingly, previous studies demonstrated that miR-181a-5p reduces invasion and tumor growth and identified kinases of the MAPK pathway as valid miR-181a-5p targets, for instance, mitogen-activated protein kinase 1 (ERK2), mitogen-activated protein kinase kinase 1 (MEK1), and the downstream transcription factor cyclic adenosine monophosphate-responsive element binding protein 1 (CREB1, Liu et al. 2013; Huang et al. 2016; Wang et al. 2017b). Especially the CREB1-miR181a-5p interaction is intriguing, as CREB1 also suppresses miR-181a-5p transcription resulting in a regulatory feedback loop (Fu et al. 2021).

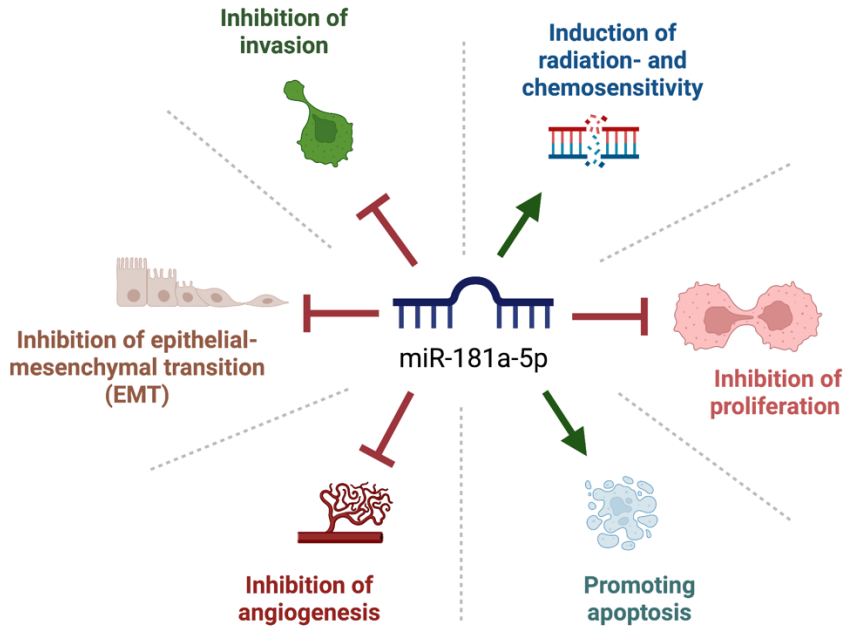

Figure 1: Role of miR-181a-5p in GBM. In GBM, miR-181a-5p is a tumor suppressor miRNA inducing apoptosis and sensitizing cells to radio-chemotherapy while inhibiting proliferation, invasion, EMT, and angiogenesis. Illustration created with biorender.com.

1.2.2 miR-425-5p

With striking oncogenic properties, miR-425-5p promotes tumor growth, invasion, metastasis, and immune evasion in human cancers, for instance, in colorectal carcinoma, non-small-cell lung cancer (NSCLC), and ovarian cancer (Wang et al. 2020; Zhou et al. 2020; Wu et al. 2021). Indeed, initial evidence also suggests that miR-425-5p is an onco-miRNA in GBM (La Rocha et al. 2020). In addition, expression levels of miR-425-5p are correlated with a poor prognosis in GBM, ovarian cancer, and breast cancer (Xiao et al. 2019; La Rocha et al. 2020; Wu et al. 2021). By targeting the tumor suppressor phosphatase and tensin homolog (PTEN), miR-425-5p accelerates PI3K/AKT signaling (Xiao et al. 2019). Furthermore, a previous study showed that miR-425-5p is highly expressed in GSCs and stabilizes the GSC phenotype by promoting neurosphere formation (La Rocha et al. 2020). However, the role of miR-425-5p in GBM remains widely unexplored.

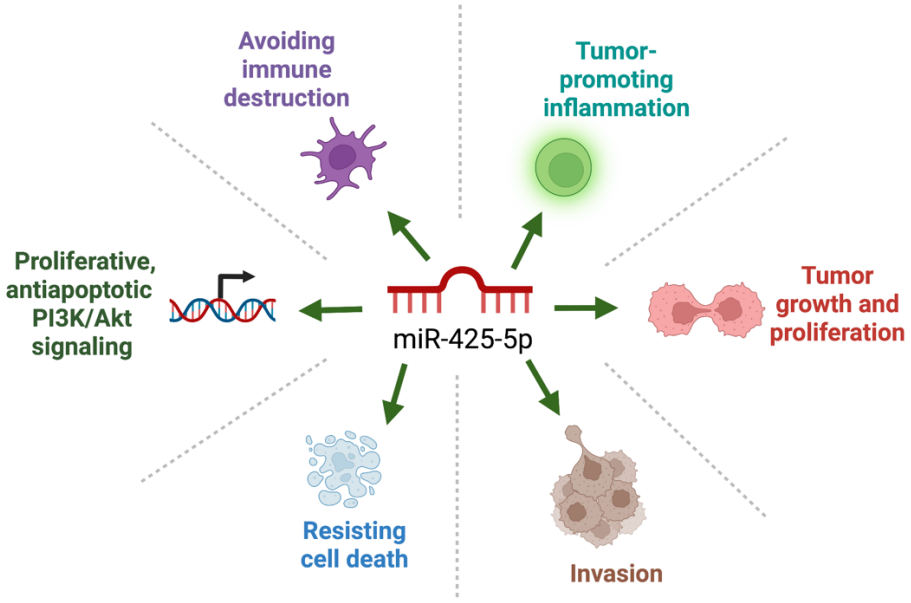

Figure 2: Role of miR-425-5p in cancer. miR-425-5p accelerates tumor progression by promoting invasion, proliferation, immune response modulation, and anti-apoptotic signaling. Illustration created with biorender.com.

1.3 A disintegrin and metalloproteinase domain-containing protein 8

A disintegrin and metalloproteinase domain-containing protein 8 (ADAM8) is a transmembrane member of the metzincin superfamily that catalyzes ECM shedding and initiates intracellular signaling (Conrad et al. 2019). After pro-domain removal, mature ADAM8 is structurally composed of a metalloprotease domain, a disintegrin domain, cysteine-rich and EGF-like domains, and a cytoplasmatic domain (Conrad et al. 2019). Physiologically, ADAM8 is selectively expressed and enriched in immune cells such as macrophages, neutrophils, and natural killer (NK) cells (Gómez-Gaviro et al. 2007; Richens et al. 2007). Moreover, under homoeostatic conditions, ADAM8 is expressed in bone and lung tissue, and weakly in neural cells (Schlomann et al. 2000; Choi et al. 2001; King et al. 2004). Also, ADAM8 has been identified to play a significant role in cancer progression (Schlomann et al. 2015). This metalloproteinase is associated with aggressive courses, and correlated with reduced survival, for instance, in GBM and breast cancer (He et al. 2012; Romagnoli et al. 2014). ADAM8 promotes invasion and tumor growth by ECM cleavage and initiates oncogenic signaling pathways, primarily by its cytoplasmatic and disintegrin domains (Conrad et al. 2019). For instance, ADAM8-induced STAT3 signaling enhanced the expression of the oncogenic sialoprotein OPN, thereby promoting angiogenesis (Li et al. 2021). Furthermore, ADAM8 was identified as crucial in mediating TMZ resistance by enhancing PI3K/AKT and MAPK signaling (Dong et al. 2015). In addition, another downstream target of ADAM8 signaling is the focal

adhesion kinase (FAK), induced by ADAM8 – integrin beta-1 (ITGB1) interaction (Schlomann et al. 2015; Awan et al. 2021). In breast cancer metastasis, a striking correlation between ADAM8 and MMP9 was further explored, and indeed ADAM8 can induce MMP9 expression via MAPK signaling, contributing to tumor aggressiveness (Conrad et al. 2018). In addition, ADAM8 was identified to modulate miRNA expression, as ADAM8-induced MAPK signaling regulated miR-720 expression in triple-negative breast cancer (Das et al. 2016).

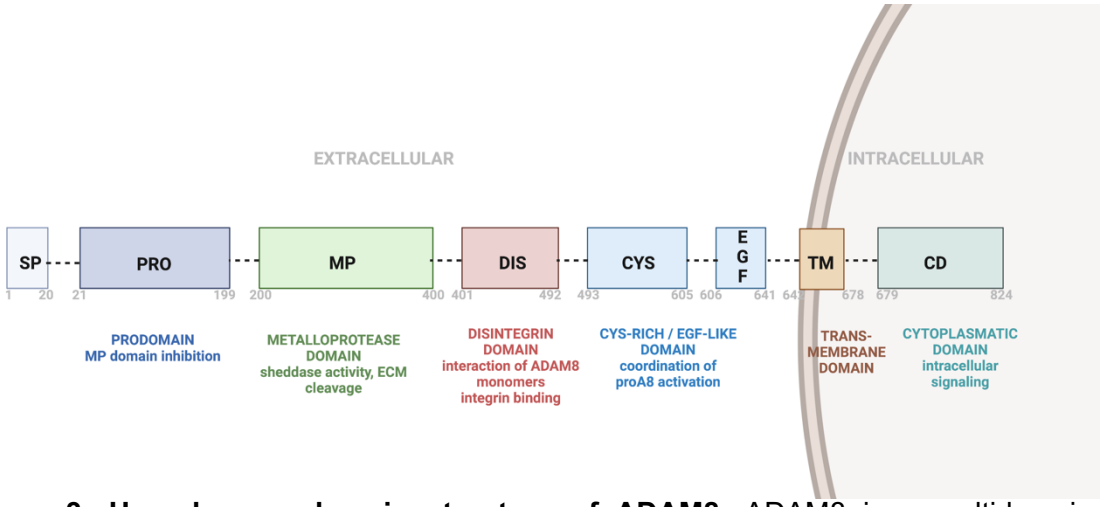

Figure 3: Homologous domain structure of ADAM8. ADAM8 is a multidomain enzyme, and after prodomain (PRO) removal, its active form is composed of a metalloprotease domain (MP), a disintegrin domain (DIS), a cysteine-rich (CYS) and EGF-like (EGF) domain, a transmembrane domain (TM) and a cytoplasmatic domain (CD). Adapted from Conrad et al. 2019, created and modified with biorender.com.

1.4 Aim of this thesis

This work aims to identify regulatory miRNAs for GBM progression. While upregulated onco-miRNAs promote GBM hallmarks like angiogenesis or tumor cell proliferation, tumor suppressor miRNAs are often downregulated. However, regulatory pathways silencing tumor suppressor miRNAs remain widely elusive. As a membrane-localized oncogene, ADAM8 induces key pathways of GBM progression, thereby mediating gene expression. For this reason, the regulatory role of ADAM8 in miRNA expression was explored. As GSCs play a pivotal role in initiating GBM recurrence and therapeutic resistance, another focus of this thesis was laid on identifying miRNAs of GSC maintenance.

2. Summary of the publications

2.1 The Metalloprotease-Disintegrin ADAM8 alters the tumor suppressor miR-181a-5p expression profile in glioblastoma thereby contributing to its aggressiveness

Agnes Schäfer *, **Lara Evers** *, Lara Meier, Uwe Schlomann, Miriam H. A. Bopp, Gian-Luca Dreizner, Olivia Lassmann, Aaron Ben Bacha, Andreea-Cristina Benescu, Mirza Pojskic, Christian Preußer, Elke Pogge von Strandmann, Barbara Carl, Christopher Nimsky and Jörg W. Bartsch
*These authors have contributed equally to this work

2022 Frontiers in Oncology, DOI: 10.3389/fonc.2022.826273

2.1.1 Scientific Summary

MiRNAs, as small regulatory molecules of gene expression, are receiving rising attention as biomarkers for cancer progression (He et al. 2020). However, underlying signaling pathways inducing changes in miRNA expression remain widely elusive. ADAM8, a transmembrane-localized metalloproteinase, mediates critical hallmarks of cancer by ECM cleavage and initiation of oncogenic signaling (Mierke 2023). Initial evidence from a breast cancer study also suggests a regulatory role of ADAM8 in miRNA expression (Das et al. 2016). In this context, we explored ADAM8-dependent miRNA regulation in GBM by primarily screening the miRNA profile of two ADAM8 deficient U87 cell clones (U87_KO1 and U87_KO2) using a pathway-focused miRNA polymerase chain reaction (PCR) array. Stable knockout of the *ADAM8* gene in U87_KO1 and U87_KO2 by clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR associated protein 9 (Cas9) induced genomic editing was confirmed as ADAM8 mRNA, protein, and soluble ADAM8 were marginally expressed or undetectable (Figure 1A-C). Compared to a U87 control clone, ADAM8-deficient U87 cells expressed a distinctive miRNA pattern as depicted by a heatmap (Figure 1D). For further investigations, miR-181a-5p was selected because this miRNA was equally upregulated in U87_KO1 and U87_KO2, as validating experiments indicate (Figure 1E). Additionally, the expression patterns of both miR-181a-5p and ADAM8 mRNA were measured in several GBM cell lines, including three patient-derived GSC lines, three patient-derived primary GBM cell lines, and the commercial GBM cell lines G28, G112, and U251 (Figure 1F-G). Interestingly, miR-181a-5p was strongly expressed in GSCs and inversely correlated with ADAM8 mRNA expression (Figure 1H-J). Literature research revealed that miR-181a-5p, a known tumor suppressor in GBM progression, targets the oncogene OPN (Marisetty et al. 2020). Since OPN expression is induced by ADAM8-mediated STAT3 signaling, we hypothesized that ADAM8 inhibits miR-181a-5p expression and thereby induces OPN translation (Li et al. 2021). Therefore, the roles of two ADAM8-dependent

downstream signaling cascades, the MAPK and STAT3 pathways, were investigated to explore this hypothesis. First, further evidence of ADAM8-dependent downregulation of mir-181a-5p is presented, as the selective ADAM8 inhibitor BK-1361 affects miR-181a-5p expression in contrast to BK-94, a broad spectrum MMP-inhibitor (Figure 2A). In addition, transient re-expression of ADAM8 in U87_KO2 led to the significant downregulation of miR-181a-5p (Figure 2B). ADAM8 is a multidomain protease, and each domain executes distinct functions. Here, miR-181a-5p inhibition is associated with an intact cytoplasmatic domain (Figure 2B). ADAM8 regulation of miR-181a-5p is unidirectional, as miR-181a-5p mimic transfection did not affect ADAM8 levels (Figure 2C). Treatment of U87 cells and primary GBM cells with the STAT3-inhibitor WP1066 significantly increased miR-181a-5p levels in both cell lines, indicating a STAT3-dependent silencing of miR-181a-5p (Figure 2D-E). Less prominently, MEK1/2 inhibition increased miR-181a-5p levels in U87 cells (Supplementary Figure 4).

Not only does ADAM8 regulate OPN expression via STAT3 signaling, but ADAM8 expression is closely correlated with MMP9 expression in breast cancer metastasis (Conrad et al. 2018). As the oncogene MMP9 enhances tumor cell proliferation and invasion, high MMP9 levels are associated with aggressive tumors (Huang 2018). We explored whether ADAM8 induces MMP9 expression by suppressing the expression of miR-181a-5p. MMP9 expression and proliferative rates were significantly decreased in U87 KO_2 cells and in U87 miR-181a-5p mimic transfected cells compared to wild-type U87 cells (Figure 3A-D). Since miR-181a-5p and MMP9 expression are inversely correlated, we speculated that miR181a-5p could directly inhibit MMP9 translation by binding to MMP9 mRNA. Even though literature research and target prediction analysis did not reveal a putative binding site for MMP9 mRNA, miR-181a-5p targets proteins of the oncogenic MAPK pathway, which in turn induces MMP9 expression (Wang et al. 2017b; Fu et al. 2021). Indeed, phosphorylated ERK1/2 and CREB1 protein levels were significantly lower in miR-181a-5p mimic transfected U87 cells (Figure 3E).

Apart from directly secreted molecules, and cell-cell contacts, EVs are essential for intercellular communication, and their heterogeneous cargo includes miRNAs (Valadi et al. 2007). Isolated EVs from cellular supernatants of wild-type U87 (U87_CTRL), U87_KO1, and U87_KO2 cells were measured and further characterized by Nanoflow cytometry and Western Blot analysis (Figure 4A-C). EVs derived from U87_KO1 and U87_KO2 contained higher levels of miR-181a-5p than U87_CTRL EVs (Figure 4D). When EVs are internalized, the cargo of these nano-molecules can alter the behavior of the recipient cell (Clerici et al. 2021). EVs derived from U87_KO2, miR-181a-5p mimic transfected cells, and miR-181a-5p inhibitor-treated cells were taken up by U87_CTRL cells and induced changes in MMP9 protein expression (Supplementary Figure 9).

Finally, miR-181a-5p, ADAM8 mRNA, and MMP9 mRNA expression levels in GBM tissue samples of 22 GBM patients were analyzed by quantitative PCR (qPCR). Detailed clinical information and expression of histopathological markers regarding the patient cohort are visualized in Table 1. All data regarding GBM tumor specimens were normalized to control tissue specimens localized most distant to the tumor core. Overall, GBM tissue specimens displayed low levels of miR-181a-5p and comparably high levels of ADAM8 and MMP9 mRNA (Figure 5A, C). While ADAM8 and MMP9 mRNA expressions were strongly and positively correlated, miR-181a-5p expression was neither inversely correlated to MMP9 levels nor ADAM8 expression (Figure 5B, D). However, GBM is a highly heterogeneous tumor composed of functionally different areas (Soeda et al. 2015). Therefore, we explored the expression of MMP9 mRNA, ADAM8 mRNA, and miR-181a-5p in different tumor areas. These areas were detected and resected by MR-spectroscopy-guided surgery. In contrast to the tumor core (L4) and the tumor edges (L2,3), the highest miR-181a-5p levels were detected in non-tumorous "surgical access" tissue (L1), whereas MMP9 and ADAM8 mRNA levels were at their apex in highly vascularized and proliferative tumor zones (Figure 5E-G). In a pilot study to investigate the suitability of miR-181a-5p as a biomarker, EVs were isolated from serum specimens of three GBM patients suffering from recurrent GBM before and after each surgical resection. Interestingly, miR-181a-5p expression in serum-derived EVs decreased in recurrent GBM compared to the initial manifestation (Figure 5J). However, further investigations analyzing serum-derived EVs and miR-181a-5p in a large patient cohort are necessary to confirm the suitability of miR-181a-5p as a biomarker.

2.1.2 Description of own contribution

My contribution to preparing this publication includes data acquisition, planning the experimental design, executing experiments, performing data analysis as shown in Figure 1E-J, Figure 5A-D, and Supplementary Figure 10; designing Figure 6, assembling the information regarding the patient cohort presented in Table 1, compilation of Supplementary Table 1, and writing the manuscript draft.

2.2 Identification of Dysregulated microRNAs in Glioblastoma Stem-Like Cells

Lara Evers *, Agnes Schäfer *, Raffaella Pini, Kai Zhao, Susanne Stei, Christopher Nimsky and Jörg W. Bartsch
* These authors have contributed equally to this work

2023 Brain Sciences, DOI: 10.3390/brainsci13020350

2.2.1 Scientific Summary

At the cellular level, GSCs promote radiation- and chemoresistance and rapid relapse of GBM (Bao et al. 2006; Chen et al. 2012). Regulation of cancer-stem cell maintenance is a complex interaction of various regulatory molecules, including miRNAs (Aldaz et al. 2013; Sana et al. 2018). This study investigated the miRNA expression profile of GSCs and corresponding differentiated tumor cells. Therefore, three established, patient-derived GSC lines were stimulated to differentiate into adherent-growing astrocytic cells. Previously, the preparation of GSC lines from surgically resected tumors was described, further characterization of these cell lines by a side population analysis was successfully conducted, and typical GSC characteristics remained restored under cell culture conditions as implantation of these GSCs in nude mice induced highly infiltrative, diffuse growing glioblastomas (Hannen et al. 2019). Detailed information on the three GSC lines, including molecular-pathological features and clinical data, is depicted in Table 1. *In-vitro* GSC differentiation was stimulated by the withdrawal of bovine fibroblast growth factor (bFGF) and epidermal growth factor (EGF) and the addition of fetal calf serum (FCS). Morphologically, non-adherent, in spheroids growing GSCs differentiated into adherent growing, astrocytic tumor cells with long cellular protrusions (Figure 1a). On the mRNA level, stem cell marker CD133 was significantly higher expressed in GSCs than in differentiated cells, and conversely, upon GSC differentiation, a significant increase of GFAP mRNA was observed (Figure 1b). Furthermore, SOX2 protein expression decreased in differentiated tumor cells, while GFAP protein expression increased (Figure 1c).

Identifying changes in miRNA expression was realized by conducting a pathway-focused miRNA PCR array on pooled GSCs and differentiated cell samples. Here, heatmap and scatter plot analyses revealed 31 dysregulated miRNAs, especially ten strongly dysregulated miRNAs (Figure 2a-c). Interestingly, six upregulated miRNAs in differentiated cells belonged to the lethal-7 (let-7) miRNA family (Figure 2c). Furthermore, the most dysregulated miRNA of the PCR array, miR-17-5p, is associated with the oncogenic miR-17-92 cluster. MiRNA clusters are defined as miRNAs transcribed simultaneously from physically adjacent miRNA genes (Olive et al. 2010). Moreover, the biological functions of these miRNAs are similar and often complement

each other (Gruszka and Zakrzewska 2018). Remarkably, four miRNAs of the miR-17-92 cluster were upregulated in GSCs (Figure 2c).

As a next step, an extensive literature search focused on the role of the ten most dominantly dysregulated miRNAs of the PCR array in GBM. Furthermore, we sought to identify future mRNA targets using the target prediction tool mirRPathDB v2.0 (Table 2, (Kehl et al. 2020). Here, a focus was laid on targets involved in GSC maintenance or cancer stem cell differentiation. As a result, four promising miRNA candidates of GSC maintenance and differentiation were identified, miR-425-5p, miR-17-5p, let-7a-5p, and miR-223-3p. Analyzing the expression of these four miRNAs in all three GSC-lines separately, miR-425-5p was most consistently and significantly overexpressed (Figure 3a, b). In addition, miR-17-5p was upregulated in all three GSC lines, though to different extents (Supplementary Figure S2a). However, miR-223-3p and let-7a-5p downregulation in GSCs, when compared to the differentiated cell state, could not be confirmed in all three GSC lines (Supplementary Figure S2b, c).

MiR-425-5p was chosen for further functional analysis. Since miR-425-5p is upregulated in GSCs and displays a putative binding site for GFAP mRNA, we hypothesized that miR-425-5p stabilizes the GSC phenotype by inhibiting GFAP mRNA translation. Exploring this hypothesis, we successfully transfected two primary patient-derived GBM lines with miR-425-5p mimics (Figure 3c). As a previous study identified that miR-425-5p targets PTEN mRNA, we also investigated if transient miR-425-5p overexpression could alter PTEN expression in the two primary GBM lines (Zhou et al. 2020). However, upon miRNA mimic transfection, the GFAP and PTEN protein expression measured by Western Blot analysis were only consistently downregulated in one of the two primary GBM cell strains (Figure 3d-g).

Finally, bioinformatic Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis revealed that all ten strongly dysregulated miRNAs of the PCR array were involved in “PI3K/AKT signaling” and “Signaling pathways regulating pluripotency of stem cells” as their connected mRNA targets are depicted in a chord plot (Figure 4a, b).

2.2.2 Description of own contribution

My contribution to the preparation of this publication includes planning the experimental design, executing the experiments in Figure 1a, b, and Figure 2a-c, providing samples and data for Figure 1c, d, Figure 3a, b, and Figure 4a, b, conducting literature research for Table 2 as well as data analysis of Figure 1a-d, Figure 2a-c, Figure 3a-c and writing the manuscript draft.

3. Discussion

3.1 The Metalloprotease-Disintegrin ADAM8 alters the tumor suppressor miR-181a-5p expression profile in glioblastoma thereby contributing to its aggressiveness

GBM remains an aggressive, incurable disease with a devastating prognosis (Ostrom et al. 2020). It is anticipated that monotherapy targeting only one particular molecule has limitations as tumors develop resistance (Dymova et al. 2021). Therefore, exploring downregulated tumor suppressors, including miRNAs and underlying signaling pathways, could lead to new therapeutic strategies by targeting oncogenic pathways or making tumor cells more susceptible to current therapies. The oncogenic metalloproteinase–disintegrin ADAM8 is associated with aggressive tumors, and initial evidence suggests that this transmembrane protein can also regulate miRNA expression. For instance, two studies claimed a regulatory role of ADAM8 in miR-720-5p expression, though in different directions (Das et al. 2016; Verel-Yilmaz et al. 2021). For this reason, we hypothesized that ADAM8 also regulates miRNA expression in GBM. Indeed, a distinctive miRNA pattern was confirmed in ADAM8-deficient U87 cells. For further investigations, miR-181a-5p was selected due to its strong tumor-suppressive properties. In this context, previous studies demonstrated that overexpression of miR-181a-5p reduces invasion and tumor growth, making this tumor suppressor miRNA an exciting ADAM8 target for further analysis (Shi et al. 2008; Wang et al. 2015). Screening of the miR-181a-5p expression in different GBM cell lines revealed that miR-181a-5p was most highly expressed in GSCs, so this miRNA might be involved in GSC maintenance. However, further investigations by our group could not confirm this hypothesis as miR-181a-5p expression was not significantly altered upon GSC differentiation (Evers et al. 2023). Detailed investigation of GSCs is justified because this small but pluripotent cell population plays a crucial role in the acquaintance of therapeutic resistance and rapid relapses (Bao et al. 2006; Chen et al. 2012). Paradoxically, the oncogene ADAM8 is scarcely expressed in GSCs, and thereby, low ADAM8 levels and comparably high levels of the tumor suppressor miR-181a-5p were inversely correlated, highlighting the complex and unique GSC biology. Previously, it was demonstrated that miR-181a-5p sensitizes GBM cells to radiation (Chen et al. 2010). However, the link between high miR-181a-5p levels in GSCs and therapeutic resistance is uninvestigated. To strengthen our hypothesis that ADAM8 downregulates miR-181a-5p, thereby promoting GBM progression, we confirmed that transient ADAM8 re-expression in U87_KO2 led to decreased miR-181a-5p levels, and additionally, ADAM8 inhibitor treatment increased miR-181a-5p levels. However, miR-181a-5p mimic transfection did

not lead to changes in ADAM8 expression. To conclude, ADAM8-miR-181a-5p regulation is unidirectional and not a feedback loop. ADAM8, a multidomain transmembrane-anchored protein, promotes several hallmarks of cancer (Conrad et al. 2019). For instance, invasion is directly facilitated due to the cleavage of ECM molecules by its metalloproteinase domain but also indirectly promoted by the induction of the MAPK pathway (Schlomann et al. 2015; Conrad et al. 2018). Previous studies demonstrated that apart from MAPK signaling, other key signaling pathways, including PI3K/Akt, STAT3, and FAK-induced signaling, are also initiated by the ADAM8 cytoplasmatic and disintegrin domains (Dong et al. 2015; Schlomann et al. 2015; Awan et al. 2021; Li et al. 2021). This study confirmed that ADAM8-dependent miR-181a-5p regulation was associated with an intact cytoplasmatic domain. ADAM8-dependent miR-181a-5p regulation was most dominantly mediated by STAT3 signaling. STAT3 is considered a master of EMT, a promoter of tumor growth, and a mediator of tumor-supportive properties in the TME (Swiatek-Machado and Kaminska 2020). In GBM, the oncogene OPN promotes angiogenesis due to ADAM8-induced STAT3 signaling (Li et al. 2021). In addition, a previous study demonstrated that OPN is a direct miR-181a-5p target (Marisetty et al. 2020). In summary, we present an ADAM8-STAT3-miR-181a-5p-OPN pathway promoting GBM progression by induction of angiogenesis. Although ADAM8 signaling leads to increased STAT3 activity, constitutive STAT3 expression in GBM results from numerous hyperactive signaling pathways, including EGFR and Janus kinase 2 (JAK2) downstream signaling (Fu et al. 2023). Therefore, it cannot be excluded that STAT3 signaling could silence miR-181a-5p independently of ADAM8.

Apart from STAT3 signaling, ADAM8-induced MAPK signaling also suppressed miR-181a-5p in U87. Fu et al. demonstrated that the MAPK cascade downstream transcription factor CREB1 directly binds to the miR-181a-5p promoter region, thereby repressing miR-181a-5p transcription (Fu et al. 2021). Physiologically, MAPK signaling is induced by cytokines and growth factors, for instance, platelet-derived growth factor (PDGF) and EGF (Heldin and Lennartsson 2013). In numerous types of cancer, however, this signaling cascade is constitutively activated, facilitating crucial hallmarks of cancer like invasion and resistance to apoptotic stimuli (Guo et al. 2020). In GBM, accelerated MAPK signaling is most frequently caused by an amplified or mutated EGFR (Padfield et al. 2015). Interestingly, miR-181a-5p also directly targets the MAPK pathway, namely ERK2, MEK1, and CREB1, resulting in a complex regulatory MAPK-miR-181a-5p feedback network that deserves detailed investigation (Liu et al. 2013; Wang et al. 2017b). We demonstrated that miR-181a-5p mimic transfection only reduced phosphorylated ERK1/2 and CREB1 levels, while unphosphorylated ERK1/2 and CREB1 levels remained stable. Mechanistically, miRNAs repress mRNA translation, so

these results must be investigated further to comprehend them fully. However, similar observations for miR181d-5p were made, as this member of the miR-181 family only reduced phosphorylated levels of AKT and ERK (Wang et al. 2012). Functionally, aberrant MAPK signaling promotes uncontrolled mitosis and tumor growth, and we present that transient miR-181a-5p overexpression decreased proliferative rates in U87 cells. Furthermore, ADAM8 knockout in U87 cells and miR-181a-5p mimic transfection resulted in decreased MMP9 levels. Combining these findings, we hypothesized that miR-181a-5p directly targets MMP9 mRNA, thereby facilitating its tumor-suppressive properties. However, bioinformatic target prediction analysis of MMP9 mRNA did not reveal a binding site for miR-181a-5p. As a result, we postulate that miR-181a-5p indirectly decreases MMP9 expression by targeting the MAPK pathway.

A previous study highlighted the striking correlation between ADAM8 and MMP9 in breast cancer metastasis and unraveled that ADAM8 induces MMP9 expression by initiating MAPK signaling (Conrad et al. 2018). Moreover, high ADAM8 and MMP9 expressions correlate separately with aggressive courses and reduced survival in GBM (He et al. 2012; Li et al. 2016). This study confirmed the ADAM8-MMP9 correlation for GBM tissue specimens and demonstrated that both oncogenes were upregulated while the tumor suppressor miR-181a-5p was downregulated. Concordant to our observations, other groups also described miR-181a-5p downregulation in GBM specimens (Shi et al. 2008; Zhai et al. 2019). Pursuing the observation that miR-181a-5p expression is decreased in GBM compared to lower-grade astrocytoma, Valiulyte et al. demonstrated that increased miR-181a-5p levels not only correlated with prolonged patient survival but also with higher functional patient status and IDH mutations (Valiulyte et al. 2022). Given the above and considering that the MGMT methylation status is the only clinically established prognostic histopathological biomarker for GBM, miR-181a-5p could be further investigated for its suitability as a new independent prognostic biomarker. A hallmark of GBM is this malignancy's highly heterogenous composure (Soeda et al. 2015). Therefore, we hypothesized that the expression of tumor suppressors or oncogenes could vary widely when comparing functionally different tumor zones in one patient. Against this backdrop, the expression profiles of miR-181a-5p, ADAM8, and MMP9 were detailly investigated in different tumor zones of one GBM patient using MR-spectroscopy-guided surgery. In cancer biology, members of the metzincin family such as MMP9 or ADAM8 degrade ECM components sustaining tumor invasion and growth, and indeed, ADAM8 and MMP9 levels were at their apex in highly proliferative tumor zones (Cui et al. 2017; Conrad et al. 2019). In addition, comparing the miR-181a-5p expression in different tumor zones of one GBM patient confirmed the assumption that

this tumor suppressor miRNA is scarcely expressed in the tumor center when compared to a non-tumorous brain tissue specimen.

Besides aberrant intracellular functions, the TME promotes GBM progression through excellent tumor-supporting conditions (Da Ros et al. 2018). Therefore, tumor cells, especially GSCs, shape their environment by releasing secretory molecules and EVs (Folkins et al. 2009; Boyd et al. 2021). Analysis of U87-derived EVs revealed that ADAM8-induced miR-181a-5p downregulation extends to the EV cargo. Marisetty et al. demonstrated that miR-181a-5p is scarcely expressed in TAMs. In contrast, the miR-181a-5p target OPN was greatly expressed, promoting tumor-supportive M2 macrophage polarization (Marisetty et al. 2020). M2 polarization is characterized by high chemokine (C-C motif) ligand 13 (CCL13) expression, which positively correlates with MMP9 levels in GBM specimens (Gjorgjevski et al. 2019). In PDAC, it was demonstrated that ADAM8 is enriched in EVs and induced high MMP9 expression in the TME (Cook et al. 2022). Therefore, ADAM8-mediated miR-181a-5p downregulation could promote immunosuppression within the glioblastoma TME. Not only are EVs vehicles of TME communication but these nano-molecules are also secreted into the bloodstream. For this reason, EVs are receiving increasing attention as diagnostic biomarkers or even future therapeutic vehicles (Urabe et al. 2020). It is therefore likely that delivery of miRNA181a-5p could have a therapeutic potential in GBM by reducing the expression of genes that are essential for GBM progression. In addition, the investigation of miR-181a-5p in serum-derived EVs revealed decreased levels in recurrent GBM. These findings justify further analysis in a large patient cohort to confirm miR-181a-5p as a potential biomarker of GBM progression.

To conclude, this study explored that downregulation of the tumor suppressor miR-181a-5p by ADAM8-induced STAT3 and MAPK signaling promotes GBM progression.

3.2 Identification of Dysregulated microRNAs in Glioblastoma Stem-Like Cells

Mediated by GSCs, radiation- and chemotherapy resistance constitutes a central problem in treating patients with recurrent GBM (Bao et al. 2006; Chen et al. 2012). Identifying cancer stem cell fate regulators, including miRNAs, could unravel new therapeutic targets as differentiated GBM cells are more prone to therapy efficacy.

Previous investigations confirmed that the three investigated GSC lines maintained their stem cell typical features as implantation of GSCs in nude mice generated highly invasive tumors (Hannen et al. 2019). Morphologically, GSCs formed spheroids while the differentiated cell state developed long star-shaped cellular protrusions and grew in monolayers. Considering GBM heterogeneity, several molecular GSC markers were

investigated to verify GSC differentiation further. We demonstrated that stem cell markers SOX2 and CD133 decreased, and GFAP levels rose upon differentiation in all three investigated GSC lines, though to different extents. Due to striking phenotypic plasticity, GBM is often divided into three subtypes, the mesenchymal type, the pro-neural type, and the classical type. For instance, high CD133 levels are strongly associated with GSCs of the pro-neural and mesenchymal subtypes (Zarkoob et al. 2013). In particular, the mesenchymal subtype is associated with increased therapeutic resistance and reduced overall survival (Phillips et al. 2006). The intermediary filament GFAP is commonly used as a differentiation marker, as differentiated tumor cells show similar characteristics to astrocytes (Ricci-Vitiani et al. 2008). However, a hallmark of cancer progression is EMT, in the case of GBM, also referred to as glial-mesenchymal transition, leading to reduced GFAP expression in recurrent gliomas (Kubelt et al. 2015). This further highlights the benefits of establishing new markers of GSCs differentiation, including miRNAs, and investigating a panel of stemness and differentiation markers to characterize GSCs properly. However, *in-vitro* GSC differentiation is widely discussed due to the lack of TME stimuli. For instance, hypoxia is a vital feature of the TME, and induction of hypoxia-inducible factor (HIF) related signaling stabilizes the stem cell state (Soeda et al. 2009).

Investigating the miRNA profiles of GSCs and their corresponding differentiated cell states could uncover new regulatory mechanisms of GSC maintenance. Primary screening of GSCs and corresponding differentiated cells by PCR analysis unraveled 31 dysregulated miRNAs. A thorough investigation of the ten most dysregulated miRNAs by bioinformatic KEGG enrichment analysis uncovered that these miRNAs were most significantly associated with “PI3K/AKT signaling” and “Signaling pathways regulating pluripotency of stem cells”. Prediction of miRNA targets involved in stem cell fate revealed numerous interesting target genes, such as insulin-like growth factor 1 receptor (IGF1R). This receptor tyrosine kinase is implicated in anti-apoptotic signaling and overexpressed in GBM (Wang et al. 2017a).

Combining the research data, four promising miRNAs, miR-425-5p, miR-15-5p, miR-223-3p, and let-7a-5p, were selected for further investigations. Elevation of miR-17-5p attributes to the anti-apoptotic, highly proliferative nature of GBM, and its overexpression is associated with a dismal prognosis (Ernst et al. 2010; Gruszka and Zakrzewska 2018). In this study, miR-17-5p was overexpressed in the three GSC lines when compared to their differentiated state. Functionally, miR-17-5p is part of the miR-17-92 cluster that regulates tumor and embryonic stem cell features (Mens and Ghanbari 2018). In GSCs, the miR-17-92 cluster is described to increase proliferation and sphere formation (Ernst et al. 2010; Schraivogel et al. 2011). Previous studies demonstrated that myc, a potent

cell cycle mediator, is implicated in GSCs survival and directly activates miR-17-92 cluster transcription (O'Donnell et al. 2005; Cencioni et al. 2023). However, miR-17-5p displays an authentic binding site to myc mRNA (Kehl et al. 2020). Thus, a detailed investigation of the myc - miR-17-5p regulatory network is justified.

As validating qPCR analysis indicates, miR-425-5p is consistently upregulated in all three GSC lines. This onco-miRNA promotes invasion, tumor growth, and immune suppression in breast cancer, NSCLC, and ovarian cancer (Xiao et al. 2019; Zhou et al. 2020; Wu et al. 2021). A first investigation in GBM confirmed overexpression of miR-425-5p in GBM specimens and demonstrated that high miR-425-5p levels correlated with reduced survival (La Rocha et al. 2020). Overactivated PI3K/AKT signaling promotes resistance to apoptotic stimuli and stabilizes the GSC phenotype (Barzegar Behrooz et al. 2022). Interestingly, constitutive PI3K/AKT signaling in GBM is frequently induced due to the downregulation of the tumor suppressor PTEN, a verified target of miR-425-5p (Zhou et al. 2020; Hashemi et al. 2023). Moreover, bioinformatic target analysis predicted GFAP mRNA as a direct miR-425-5p target. Therefore, primary patient-derived differentiated GBM cells altered GFAP and PTEN expression after miR-425-5p mimic transfection. EMT is a cornerstone of cancer progression, and a previous study demonstrated that miR-425-5p overexpression drives mesenchymal transformation in hepatocellular carcinoma (Fang et al. 2017). In GBM, glial-mesenchymal transition is linked to reduced GFAP expression and is crucial for GSC maintenance (Bhat et al. 2011; Kubelt et al. 2015). Therefore, miR-425-5p could stabilize the GSC phenotype and promote mesenchymal transformation by downregulating GFAP expression and accelerating PI3K/Akt signaling in GBM.

We detected that GSC differentiation induced the upregulation of nine miRNAs, including six members of the let-7-mirRNA family and miR-223-3p. On the one hand, the let-7-miRNA family displays tumor-suppressive characteristics in GBM by targeting rat sarcoma virus (RAS), thereby silencing the MAPK pathway and inhibiting migration and proliferation (Wang et al. 2013). On the other hand, a previous study demonstrated that miR-223-3p could enhance radiation sensitivity by targeting ataxia-telangiectasia mutated (ATM, Liang et al. 2014). This protein is overexpressed in GSCs and promotes multi-resistant relapses, but the role of miR-223 in GSCs is unexplored (Li et al. 2017). In addition, target prediction analysis uncovered that miR-223-3p harbors a putative binding site for MMP9 mRNA. Thus, miR-223-3p could act as a tumor suppressor by repressing MMP9-induced invasion and by reducing radiation- and chemotherapy resistance. However, proceeding analyses did not confirm the upregulation of either let-7a-5p or miR223-3p in differentiated cells of all three GSC lines. GBM is a highly heterogenous malignancy, and Li et al. introduced distinctive miRNA signatures in

different GBM subtypes so that differential miR-223-3p and let-7a-5p levels could also result from GBM's remarkable phenotypic plasticity (Li et al. 2014). Supporting this hypothesis, literature research revealed evidence that high let-7 expression drives GSC differentiation consistent with investigations in other types of cancer (Song et al. 2016; Ma et al. 2021). By bioinformatic target prediction, we identified that Musashi-2 (MSI2) displays putative binding sites for miR223-3p and several let-7-members, including let-7a-5p. MSI2 is associated with SOX2 expression and, thereby, essential for maintaining self-renewal capacities in stem cells (Wuebben et al. 2012). Hence, further explorations of the let-7 family and miR-223-3p in GBM, especially GSCs, seem justified.

In summary, this study detected changes in the miRNA profile of GSCs and their corresponding differentiated cell state and identified that miR-425-5p, miR-17-5p, miR-223-3p, and let-7a-5p are linked to GSC maintenance and tumor progression.

4. Summary

As a highly invasive and progressive tumor, GBM ranks among the most lethal cancers. MiRNAs, as small regulators of posttranscriptional gene expression, are associated with aberrant intracellular signaling pathways that characterize GBM biology. Recurrent, progressive GBM is hardly treatable due to limited therapeutic options and the rapid acquaintance of radiation- and chemotherapy resistance. These relapses are often mediated by GSCs, a small but highly influential fraction of tumor cells with pluripotent properties. Identifying mediators of GBM progression, GCS maintenance, and underlying dysregulated signaling pathways is essential for developing new therapeutic strategies. This thesis investigated the role of miRNAs in GBM progression by identifying miRNAs of GSC maintenance and by exploring signaling pathways downregulating miR-181a-5p, a tumor suppressor miRNA in GBM.

The membrane-anchored ADAM8 induces the transcription of pro-invasive and angiogenic oncogenes by initiating intracellular signaling pathways. In this context, the first study uncovered a new regulatory network implementing miR-181a-5p downregulation by ADAM8-induced MAPK and STAT3 signaling. In ADAM8-deficient U87 cells, miR-181a-5p was significantly upregulated. Notably, the overexpression of miR-181a-5p extended to the EV cargo. The cytoplasmatic domain of ADAM8 accelerates MAPK and STAT3 signaling, thereby silencing miR-181a-5p transcription. Furthermore, transiently overexpressed miR-181a-5p reduced proliferation and MMP9 expression by targeting MAPK pathway kinases, highlighting this miRNA's tumor-suppressive properties, and introducing a complex regulatory miR-181a-5p/MAPK/MMP9 loop. In tissue specimens ADAM8 and MMP9 were overexpressed, their levels were strikingly correlated, while miR-181a-5p was downregulated. Analysis of patient-serum-derived EVs revealed reduced miR-181a-5p levels in recurrent glioblastoma proposing miR-181a-5p as a potential biomarker of GBM progression.

The second study explored the miRNA profiles of GSCs and their corresponding differentiated cell states, aiming to uncover regulatory miRNAs of GSC maintenance. Upon *in-vitro* differentiation of well-characterized GSCs into astrocytic tumor cells, 31 dysregulated miRNAs were uncovered by conducting a miRNA PCR array. Combing literature research and bioinformatic analyses, four miRNAs, miR-425-5p, miR-223-3p, let-7a-5p, and miR-17-5p, were introduced as putative mediators of GSC fate. Since target prediction analysis revealed that PTEN and GFAP mRNAs display authentic binding sites for miR-425-5p, and validating experiments confirmed miR-425-5p overexpression in all GSC lines, this onco-miRNA was selected for further investigations. Indeed, GFAP and PTEN expression decreased upon miR-425-5p overexpression in

one primary patient-derived GBM cell line, highlighting a potential role of this onco-miRNA in GSC survival and GBM progression.

A distinctive miRNA profile is linked to core features of GBM like invasion, therapeutic resistance, and phenotype plasticity. Thereby, altered expressions of numerous miRNAs, including miR-425-5p and miR-181-5p, are associated with GBM progression and discussed as future biomarkers of GBM recurrence.

5. Zusammenfassung

Das GBM ist eine primär hirneigene, hoch invasive Neoplasie mit fataler Prognose. Als zentrale Mediatoren der post-transkriptionellen Genexpression regulieren miRNAs kritische Signalwegen des GBM. Die ausgeprägte Rezidivneigung und die rapide Entwicklung von Resistenzen gegenüber der adjuvanten Therapie, stellen zentrale Probleme in der Behandlung des GBM dar. Bedeutende Mediatoren dieser fatalen Tumoreigenschaften sind GSCs, eine kleine Tumorzellfraktion mit stammzellähnlichen Charakteristiken. Ziel dieser Arbeit ist es, miRNAs als potenzielle Marker und Regulatoren für die Progression des GBM zu analysieren. Unter Berücksichtigung der einzigartigen Rolle von GSCs wurde das miRNA Profil dieser Tumorzellfraktion differenziert untersucht. Zudem wurden die Hintergründe und Konsequenzen der verminderten Expression von miR-181a-5p, einer Tumorsuppressor miRNA im GBM, detailliert exploriert.

ADAM8 ist eine Disintegrin-Metalloproteinase, die durch Initiation von intrazellulären Signalwegen die Expression von weiteren Onkogenen fördern kann. Vor diesem Hintergrund ergründete die erste vorgestellte Studie die Zusammenhänge zwischen ADAM8-induzierter, onkologischer Signalgebung und der verminderten miR-181a-5p Expression im GBM. Die miR-181a-5p Expression zeigte sich nicht nur in ADAM8-Knockout U87 Zellen erhöht, sondern auch in EVs dieser Zellen. Der verminderten miR-181a-5p Expression in U87 Zellen lag die ADAM8-induzierte gesteigerte Stimulation von den MAPK- und STAT3- Signalwegen zugrunde. Diese Studie demaskierte ein komplexes miR-181a-5p/MAPK/MMP9 Netzwerk, da die transiente Überexpression von miR-181a-5p zu verminderter Zellproliferation und erniedrigter MMP9 Expression führte. In GBM-Gewebeproben zeigten die stark exprimierten Onkogene ADAM8 und MMP9 eine positive Korrelation. Gegensätzlich war miR-181a-5p nur niedrig exprimiert. Um die Eignung von miR-181a-5p als potenziellen Biomarker zu prüfen, analysierten wir EVs aus Serumproben von drei Patienten mit Glioblastomrezidiven und zeigten, dass die miR-181a-5p Expression mit dem Krankheitsprogress sinkt.

In der zweiten Studie wurde das miRNA-Profil von GSCs und ihrem differenzierten Status untersucht, um die regulatorische Rolle von miRNAs auf Tumorstammzelleigenschaften zu beleuchten. Mit der Ausdifferenzierung von GSCs zu astrozytären Tumorzellen, änderte sich die miRNA Expression deutlich, insgesamt wurden 31 dysregulierte miRNAs entdeckt. Unter Berücksichtigung von Literaturrecherchen und bioinformatischer Analysen wurden vier miRNAs miR-425-5p, miR-223-3p, let-7a-5p und miR-17-5p als potenzielle Mediatoren von GSC-Eigenschaften vorgestellt und weitergehend untersucht. Am eindeutigsten stellte sich die Überexpression von miR-425-5p in GSCs dar. In einer primären Glioblastomzelllinie

führte die transiente Überexpression von miR-425-5p zu erniedrigten Expressionen von PTEN und GFAP, deren mRNA-Sequenzen potenzielle Ziele dieser onco-miRNA sind. Ein verändertes miRNA-Expressionsprofil ist mit Kernmerkmalen des Glioblastoms wie Invasion, therapeutischer Resistenz und Tumorheterogenität assoziiert. Da die veränderte Expression zahlreicher miRNAs, einschließlich miR-425-5p und miR-181-5p, eng mit der Glioblastomprogression assoziiert ist, werden miRNAs als zukünftige Biomarker für Glioblastomrezidive diskutiert.

6. References

Aghajani M, Mansoori B, Mohammadi A, Asadzadeh Z, Baradaran B, 2019. New emerging roles of CD133 in cancer stem cell: Signaling pathway and miRNA regulation. Journal of cellular physiology, 234 (12), 21642–21661.

Aldaz B, Sagardoy A, Nogueira L, Guruceaga E, Grande L, Huse JT, Aznar MA, Díez-Valle R, Tejada-Solís S, Alonso MM, Fernandez-Luna JL, Martinez-Climent JA, Malumbres R, 2013. Involvement of miRNAs in the differentiation of human glioblastoma multiforme stem-like cells. PloS one, 8 (10), e77098.

Ali Syeda Z, Langden SSS, Munkhzul C, Lee M, Song SJ, 2020. Regulatory Mechanism of MicroRNA Expression in Cancer. International journal of molecular sciences, 21 (5).

Anjum K, Shagufta BI, Abbas SQ, Patel S, Khan I, Shah SAA, Akhter N, Hassan SSU, 2017. Current status and future therapeutic perspectives of glioblastoma multiforme (GBM) therapy: A review. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie, 92, 681–689.

Awan T, Babendreyer A, Mahmood Alvi A, Düsterhöft S, Lambertz D, Bartsch JW, Liedtke C, Ludwig A, 2021. Expression levels of the metalloproteinase ADAM8 critically regulate proliferation, migration and malignant signalling events in hepatoma cells. Journal of cellular and molecular medicine, 25 (4), 1982–1999.

Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD, Rich JN, 2006. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature, 444 (7120), 756–760.

Barzegar Behrooz A, Talaie Z, Jusheghani F, Łos MJ, Klonisch T, Ghavami S, 2022. Wnt and PI3K/Akt/mTOR Survival Pathways as Therapeutic Targets in Glioblastoma. International journal of molecular sciences, 23 (3).

Bhat KPL, Salazar KL, Balasubramaniyan V, Wani K, Heathcock L, Hollingsworth F, James JD, Gumin J, Diefes KL, Kim SH, Turski A, Azodi Y, Yang Y, Doucette T, Colman H, Sulman EP, Lang FF, Rao G, Copray S, Vaillant BD, Aldape KD, 2011. The transcriptional coactivator TAZ regulates mesenchymal differentiation in malignant glioma. Genes & development, 25 (24), 2594–2609.

Boyd NH, Tran AN, Bernstock JD, Etminan T, Jones AB, Gillespie GY, Friedman GK, Hjelmeland AB, 2021. Glioma stem cells and their roles within the hypoxic tumor microenvironment. Theranostics, 11 (2), 665–683.

Bulik M, Jancalek R, Vanicek J, Skoch A, Mechl M, 2013. Potential of MR spectroscopy for assessment of glioma grading. Clinical neurology and neurosurgery, 115 (2), 146–153.

Buruiană A, Florian ȘI, Florian AI, Timiș T-L, Mihu CM, Miclăuș M, Oșan S, Hrapșa I, Cataniciu RC, Farcaș M, Șușman S, 2020. The Roles of miRNA in Glioblastoma Tumor Cell Communication: Diplomatic and Aggressive Negotiations. International journal of molecular sciences, 21 (6).

Butler M, Pongor L, Su Y-T, Xi L, Raffeld M, Quezado M, Trepel J, Aldape K, Pommier Y, Wu J, 2020. MGMT Status as a Clinical Biomarker in Glioblastoma. Trends in cancer, 6 (5), 380–391.

Cavallari C, Camussi G, Brizzi MF, 2020. Extracellular Vesicles in the Tumour Microenvironment: Eclectic Supervisors. International journal of molecular sciences, 21 (18).

Cencioni C, Scagnoli F, Spallotta F, Nasi S, Illi B, 2023. The "Superoncogene" Myc at the Crossroad between Metabolism and Gene Expression in Glioblastoma Multiforme. International journal of molecular sciences, 24 (4).

Chen G, Zhu W, Shi D, Lv L, Zhang C, Liu P, Hu W, 2010. MicroRNA-181a sensitizes human malignant glioma U87MG cells to radiation by targeting Bcl-2. Oncology Reports, 23 (4).

Chen J, Li Y, Yu T-S, McKay RM, Burns DK, Kernie SG, Parada LF, 2012. A restricted cell population propagates glioblastoma growth after chemotherapy. Nature, 488 (7412), 522–526.

Chinot OL, Wick W, Mason W, Henriksson R, Saran F, Nishikawa R, Carpentier AF, Hoang-Xuan K, Kavan P, Cernea D, Brandes AA, Hilton M, Abrey L, Cloughesy T, 2014. Bevacizumab plus radiotherapy-temozolomide for newly diagnosed glioblastoma. The New England journal of medicine, 370 (8), 709–722.

Choi SJ, Han JH, Roodman GD, 2001. ADAM8: a novel osteoclast stimulating factor. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research, 16 (5), 814–822.

Clerici SP, Peppelenbosch M, Fuhler G, Consonni SR, Ferreira-Halder CV, 2021. Colorectal Cancer Cell-Derived Small Extracellular Vesicles Educate Human Fibroblasts to Stimulate Migratory Capacity. Frontiers in cell and developmental biology, 9, 696373.

Codrici E, Popescu I-D, Tanase C, Enciu A-M, 2022. Friends with Benefits: Chemokines, Glioblastoma-Associated Microglia/Macrophages, and Tumor Microenvironment. International journal of molecular sciences, 23 (5).

Conrad C, Benzel J, Dorzweiler K, Cook L, Schlomann U, Zarbock A, Slater EP, Nimsky C, Bartsch JW, 2019. ADAM8 in invasive cancers: links to tumor progression, metastasis, and chemoresistance. Clinical science (London, England : 1979), 133 (1), 83–99.

Conrad C, Götte M, Schlomann U, Roessler M, Pagenstecher A, Anderson P, Preston J, Pruessmeyer J, Ludwig A, Li R, Kamm RD, Ritz R, Carl B, Nimsky C, Bartsch JW, 2018. ADAM8 expression in breast cancer derived brain metastases: Functional implications on MMP-9 expression and transendothelial migration in breast cancer cells. International journal of cancer, 142 (4), 779–791.

Cook L, Sengelmann M, Winkler B, Nagl C, Koch S, Schlomann U, Slater EP, Miller MA, Strandmann EP von, Dörsam B, Preußer C, Bartsch JW, 2022. ADAM8-Dependent Extracellular Signaling in the Tumor Microenvironment Involves Regulated Release of Lipocalin 2 and MMP-9. International journal of molecular sciences, 23 (4).

Cui N, Hu M, Khalil RA, 2017. Biochemical and Biological Attributes of Matrix Metalloproteinases. Progress in molecular biology and translational science, 147, 1–73.

Da Ros M, Gregorio V de, Iorio A, Giunti L, Guidi M, Martino M de, Genitori L, Sardi I, 2018. Glioblastoma Chemoresistance: The Double Play by Microenvironment and Blood-Brain Barrier. International Journal of Molecular Sciences, 19 (10), 2879.

Dapash M, Hou D, Castro B, Lee-Chang C, Lesniak MS, 2021. The Interplay between Glioblastoma and Its Microenvironment. Cells, 10 (9).

Das SG, Romagnoli M, Mineva ND, Barillé-Nion S, Jézéquel P, Campone M, Sonenshein GE, 2016. miR-720 is a downstream target of an ADAM8-induced ERK signaling cascade that promotes the migratory and invasive phenotype of triple-negative breast cancer cells. Breast cancer research : BCR, 18 (1), 40.

Dong F, Eibach M, Bartsch JW, Dolga AM, Schlomann U, Conrad C, Schieber S, Schilling O, Biniossek ML, Culmsee C, Strik H, Koller G, Carl B, Nimsky C, 2015. The metalloprotease-disintegrin ADAM8 contributes to temozolomide chemoresistance and enhanced invasiveness of human glioblastoma cells. Neuro-oncology, 17 (11), 1474–1485.

Dymova MA, Kuligina EV, Richter VA, 2021. Molecular Mechanisms of Drug Resistance in Glioblastoma. International journal of molecular sciences, 22 (12).

Ernst A, Campos B, Meier J, Devens F, Liesenberg F, Wolter M, Reifenberger G, Herold-Mende C, Lichter P, Radlwimmer B, 2010. De-repression of CTGF via the miR-17-

92 cluster upon differentiation of human glioblastoma spheroid cultures. Oncogene, 29 (23), 3411–3422.

Evers L, Schäfer A, Pini R, Zhao K, Stei S, Nimsky C, Bartsch JW, 2023. Identification of Dysregulated microRNAs in Glioblastoma Stem-like Cells. Brain sciences, 13 (2).

Fabian MR, Sonenberg N, Filipowicz W, 2010. Regulation of mRNA translation and stability by microRNAs. Annual review of biochemistry, 79, 351–379.

Fang F, Song T, Zhang T, Cui Y, Zhang G, Xiong Q, 2017. MiR-425-5p promotes invasion and metastasis of hepatocellular carcinoma cells through SCAI-mediated dysregulation of multiple signaling pathways. Oncotarget, 8 (19), 31745–31757.

Folkins C, Shaked Y, Man S, Tang T, Lee CR, Zhu Z, Hoffman RM, Kerbel RS, 2009. Glioma tumor stem-like cells promote tumor angiogenesis and vasculogenesis via vascular endothelial growth factor and stromal-derived factor 1. Cancer research, 69 (18), 7243–7251.

Fu W, Hou X, Dong L, Hou W, 2023. Roles of STAT3 in the pathogenesis and treatment of glioblastoma. Frontiers in cell and developmental biology, 11, 1098482.

Fu Y, Xin Z, Ling Z, Xie H, Xiao T, Shen X, Lin J, Xu L, Jiang H, 2021. A CREB1-miR-181a-5p loop regulates the pathophysiologic features of bone marrow stromal cells in fibrous dysplasia of bone. Molecular medicine (Cambridge, Mass.), 27 (1), 81.

Garnier D, Renoult O, Alves-Guerra M-C, Paris F, Pecqueur C, 2019. Glioblastoma Stem-Like Cells, Metabolic Strategy to Kill a Challenging Target. Frontiers in oncology, 9, 118.

Gimple RC, Bhargava S, Dixit D, Rich JN, 2019. Glioblastoma stem cells: lessons from the tumor hierarchy in a lethal cancer. Genes & development, 33 (11-12), 591–609.

Gjorgjevski M, Hannen R, Carl B, Li Y, Landmann E, Buchholz M, Bartsch JW, Nimsky C, 2019. Molecular profiling of the tumor microenvironment in glioblastoma patients: correlation of microglia/macrophage polarization state with metalloprotease expression profiles and survival. Bioscience reports, 39 (6).

Gómez-Gaviro M, Domínguez-Luis M, Canchado J, Calafat J, Janssen H, Lara-Pezzi E, Fourie A, Tugores A, Valenzuela-Fernández A, Mollinedo F, Sánchez-Madrid F, Díaz-González F, 2007. Expression and regulation of the metalloproteinase ADAM-8 during human neutrophil pathophysiological activation and its catalytic activity on L-selectin shedding. Journal of immunology (Baltimore, Md. : 1950), 178 (12), 8053–8063.

Green D, Dalmay T, Chapman T, 2016. Microguards and micromessengers of the genome. Heredity, 116 (2), 125–134.

Gritsch S, Batchelor TT, Gonzalez Castro LN, 2022. Diagnostic, therapeutic, and prognostic implications of the 2021 World Health Organization classification of tumors of the central nervous system. Cancer, 128 (1), 47–58.

Gruszka R, Zakrzewska M, 2018. The Oncogenic Relevance of miR-17-92 Cluster and Its Paralogous miR-106b-25 and miR-106a-363 Clusters in Brain Tumors. International journal of molecular sciences, 19 (3).

Guo Y-J, Pan W-W, Liu S-B, Shen Z-F, Xu Y, Hu L-L, 2020. ERK/MAPK signalling pathway and tumorigenesis. Experimental and therapeutic medicine, 19 (3), 1997–2007.

Hannen R, Selmansberger M, Hauswald M, Pagenstecher A, Nist A, Stiewe T, Acker T, Carl B, Nimsky C, Bartsch JW, 2019. Comparative Transcriptomic Analysis of Temozolomide Resistant Primary GBM Stem-Like Cells and Recurrent GBM Identifies Up-Regulation of the Carbonic Anhydrase CA2 Gene as Resistance Factor. Cancers, 11 (7).

Hashemi M, Etemad S, Rezaei S, Ziaolhagh S, Rajabi R, Rahmanian P, Abdi S, Koohpar ZK, Rafiei R, Raei B, Ahmadi F, Salimimoghadam S, Aref AR, Zandieh MA, Entezari M, Taheriazam A, Hushmandi K, 2023. Progress in targeting PTEN/PI3K/Akt axis in glioblastoma therapy: Revisiting molecular interactions. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie, 158, 114204.

He B, Zhao Z, Cai Q, Zhang Y, Zhang P, Shi S, Xie H, Peng X, Yin W, Tao Y, Wang X, 2020. miRNA-based biomarkers, therapies, and resistance in Cancer. International journal of biological sciences, 16 (14), 2628–2647.

He S, Ding L, Cao Y, Li G, Deng J, Tu Y, Wang B, 2012. Overexpression of a disintegrin and metalloprotease 8 in human gliomas is implicated in tumor progression and prognosis. Medical oncology (Northwood, London, England), 29 (3), 2032–2037.

Heldin C-H, Lennartsson J, 2013. Structural and functional properties of platelet-derived growth factor and stem cell factor receptors. Cold Spring Harbor perspectives in biology, 5 (8), a009100.

Huang H, 2018. Matrix Metalloproteinase-9 (MMP-9) as a Cancer Biomarker and MMP-9 Biosensors: Recent Advances. Sensors (Basel, Switzerland), 18 (10).

Huang X, Schwind S, Santhanam R, Eisfeld A-K, Chiang C-L, Lankenau M, Yu B, Hoellerbauer P, Jin Y, Tarighat SS, Khalife J, Walker A, Perrotti D, Bloomfield CD, Wang H, Lee RJ, Lee LJ, Marcucci G, 2016. Targeting the RAS/MAPK pathway with miR-181a in acute myeloid leukemia. Oncotarget, 7 (37), 59273–59286.

Indrieri A, Carrella S, Carotenuto P, Banfi S, Franco B, 2020. The Pervasive Role of the miR-181 Family in Development, Neurodegeneration, and Cancer. International journal of molecular sciences, 21 (6).

Kehl T, Kern F, Backes C, Fehlmann T, Stöckel D, Meese E, Lenhof H-P, Keller A, 2020. miRPathDB 2.0: a novel release of the miRNA Pathway Dictionary Database. Nucleic acids research, 48 (D1), D142-D147.

King NE, Zimmermann N, Pope SM, Fulkerson PC, Nikolaidis NM, Mishra A, Witte DP, Rothenberg ME, 2004. Expression and regulation of a disintegrin and metalloproteinase (ADAM) 8 in experimental asthma. American journal of respiratory cell and molecular biology, 31 (3), 257–265.

Kubelt C, Hattermann K, Sebens S, Mehdorn HM, Held-Feindt J, 2015. Epithelial-to-mesenchymal transition in paired human primary and recurrent glioblastomas. International journal of oncology, 46 (6), 2515–2525.

La Rocha AMA de, González-Huarriz M, Guruceaga E, Mihelson N, Tejada-Solís S, Díez-Valle R, Martínez-Vélez N, Fueyo J, Gomez-Manzano C, Alonso MM, Laterra J, López-Bertoni H, 2020. miR-425-5p, a SOX2 target, regulates the expression of FOXJ3 and RAB31 and promotes the survival of GSCs. Archives of clinical and biomedical research, 4 (3), 221–238.

Lakka SS, Jasti SL, Gondi C, Boyd D, Chandrasekar N, Dinh DH, Olivero WC, Gujrati M, Rao JS, 2002. Downregulation of MMP-9 in ERK-mutated stable transfectants inhibits glioma invasion in vitro. Oncogene, 21 (36), 5601–5608.

Lathia JD, Mack SC, Mulkearns-Hubert EE, Valentim CLL, Rich JN, 2015. Cancer stem cells in glioblastoma. Genes & development, 29 (12), 1203–1217.

Li Q, Chen B, Cai J, Sun Y, Wang G, Li Y, Li R, Feng Y, Han B, Li J, Tian Y, Yi L, Jiang C, 2016. Comparative Analysis of Matrix Metalloproteinase Family Members Reveals That MMP9 Predicts Survival and Response to Temozolomide in Patients with Primary Glioblastoma. PloS one, 11 (3), e0151815.

Li R, Gao K, Luo H, Wang X, Shi Y, Dong Q, Luan W, You Y, 2014. Identification of intrinsic subtype-specific prognostic microRNAs in primary glioblastoma. Journal of experimental & clinical cancer research : CR, 33 (1), 9.

Li Y, Guo S, Zhao K, Conrad C, Driescher C, Rothbart V, Schlomann U, Guerreiro H, Bopp MH, König A, Carl B, Pagenstecher A, Nimsky C, Bartsch JW, 2021. ADAM8 affects glioblastoma progression by regulating osteopontin-mediated angiogenesis. Biological chemistry, 402 (2), 195–206.

Li Y, Li L, Wu Z, Wang L, Wu Y, Li D, Ma U, Shao J, Yu H, Wang D, 2017. Silencing of ATM expression by siRNA technique contributes to glioma stem cell radiosensitivity in vitro and in vivo. Oncology reports, 38 (1), 325–335.

Liang L, Zhu J, Zaorsky NG, Deng Y, Wu X, Liu Y, Liu F, Cai G, Gu W, Shen L, Zhang Z, 2014. MicroRNA-223 enhances radiation sensitivity of U87MG cells in vitro and in vivo by targeting ataxia telangiectasia mutated. International journal of radiation oncology, biology, physics, 88 (4), 955–960.

Liu Y, Zhao Z, Yang F, Gao Y, Song J, Wan Y, 2013. microRNA-181a is involved in insulin-like growth factor-1-mediated regulation of the transcription factor CREB1. Journal of neurochemistry, 126 (6), 771–780.

Ma Y, Shen N, Wicha MS, Luo M, 2021. The Roles of the Let-7 Family of MicroRNAs in the Regulation of Cancer Stemness. Cells, 10 (9).

Marisetty A, Wei J, Kong L-Y, Ott M, Fang D, Sabbagh A, Heimberger AB, 2020. MiR-181 Family Modulates Osteopontin in Glioblastoma Multiforme. Cancers, 12 (12).

McKinnon C, Nandhabalan M, Murray SA, Plaha P, 2021. Glioblastoma: clinical presentation, diagnosis, and management. BMJ (Clinical research ed.), 374, n1560.

Mens MMJ, Ghanbari M, 2018. Cell Cycle Regulation of Stem Cells by MicroRNAs. Stem cell reviews and reports, 14 (3), 309–322.

Mierke CT, 2023. The versatile roles of ADAM8 in cancer cell migration, mechanics, and extracellular matrix remodeling. Frontiers in cell and developmental biology, 11, 1130823.

Møller HG, Rasmussen AP, Andersen HH, Johnsen KB, Henriksen M, Duroux M, 2013. A systematic review of microRNA in glioblastoma multiforme: micro-modulators in the mesenchymal mode of migration and invasion. Molecular neurobiology, 47 (1), 131–144.

Nazimek K, Bryniarski K, 2020. Perspectives in Manipulating EVs for Therapeutic Applications: Focus on Cancer Treatment. International journal of molecular sciences, 21 (13).

O'Brien J, Hayder H, Zayed Y, Peng C, 2018. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. Frontiers in endocrinology, 9, 402.

O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT, 2005. c-Myc-regulated microRNAs modulate E2F1 expression. Nature, 435 (7043), 839–843.

Olive V, Jiang I, He L, 2010. mir-17-92, a cluster of miRNAs in the midst of the cancer network. The international journal of biochemistry & cell biology, 42 (8), 1348–1354.

Ostrom QT, Patil N, Cioffi G, Waite K, Kruchko C, Barnholtz-Sloan JS, 2020. CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2013-2017. Neuro-oncology, 22 (12 Suppl 2), iv1-iv96.

Padfield E, Ellis HP, Kurian KM, 2015. Current Therapeutic Advances Targeting EGFR and EGFRvIII in Glioblastoma. Frontiers in oncology, 5, 5.

Philips A, Henshaw DL, Lamburn G, O'Carroll MJ, 2018. Brain Tumours: Rise in Glioblastoma Multiforme Incidence in England 1995-2015 Suggests an Adverse Environmental or Lifestyle Factor. Journal of environmental and public health, 2018, 7910754.

Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD, Misra A, Nigro JM, Colman H, Soroceanu L, Williams PM, Modrusan Z, Feuerstein BG, Aldape K, 2006. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. Cancer cell, 9 (3), 157–173.

Pires-Afonso Y, Niclou SP, Michelucci A, 2020. Revealing and Harnessing Tumour-Associated Microglia/Macrophage Heterogeneity in Glioblastoma. International journal of molecular sciences, 21 (3).

Priester M, Copanaki E, Vafaizadeh V, Hensel S, Bernreuther C, Glatzel M, Seifert V, Groner B, Kögel D, Weissenberger J, 2013. STAT3 silencing inhibits glioma single cell infiltration and tumor growth. Neuro-oncology, 15 (7), 840–852.

Ricci-Vitiani L, Pallini R, Larocca LM, Lombardi DG, Signore M, Pierconti F, Petrucci G, Montano N, Maira G, Maria R de, 2008. Mesenchymal differentiation of glioblastoma stem cells. Cell death and differentiation, 15 (9), 1491–1498.

Richens J, Fairclough L, Ghaemmaghami AM, Mahdavi J, Shakib F, Sewell HF, 2007. The detection of ADAM8 protein on cells of the human immune system and the demonstration of its expression on peripheral blood B cells, dendritic cells and monocyte subsets. Immunobiology, 212 (1), 29–38.

Romagnoli M, Mineva ND, Polmear M, Conrad C, Srinivasan S, Loussouarn D, Barillé-Nion S, Georgakoudi I, Dagg Á, McDermott EW, Duffy MJ, McGowan PM, Schlomann U, Parsons M, Bartsch JW, Sonenshein GE, 2014. ADAM8 expression in invasive breast cancer promotes tumor dissemination and metastasis. EMBO molecular medicine, 6 (2), 278–294.

Sana J, Busek P, Fadrus P, Besse A, Radova L, Vecera M, Reguli S, Stollinova Sromova L, Hilser M, Lipina R, Lakomy R, Kren L, Smrcka M, Sedo A, Slaby O, 2018.

Identification of microRNAs differentially expressed in glioblastoma stem-like cells and their association with patient survival. Scientific reports, 8 (1), 2836.

Schlomann U, Koller G, Conrad C, Ferdous T, Golfi P, Garcia AM, Höfling S, Parsons M, Costa P, Soper R, Bossard M, Hagemann T, Roshani R, Sewald N, Ketchem RR, Moss ML, Rasmussen FH, Miller MA, Lauffenburger DA, Tuveson DA, Nimsky C, Bartsch JW, 2015. ADAM8 as a drug target in pancreatic cancer. Nature communications, 6, 6175.

Schlomann U, Rathke-Hartlieb S, Yamamoto S, Jockusch H, Bartsch JW, 2000. Tumor necrosis factor alpha induces a metalloprotease-disintegrin, ADAM8 (CD 156): implications for neuron-glia interactions during neurodegeneration. The Journal of neuroscience : the official journal of the Society for Neuroscience, 20 (21), 7964–7971.

Schraivogel D, Weinmann L, Beier D, Tabatabai G, Eichner A, Zhu JY, Anton M, Sixt M, Weller M, Beier CP, Meister G, 2011. CAMTA1 is a novel tumour suppressor regulated by miR-9/9* in glioblastoma stem cells. The EMBO journal, 30 (20), 4309–4322.

Shea A, Harish V, Afzal Z, Chijioke J, Kedir H, Dusmatova S, Roy A, Ramalinga M, Harris B, Blancato J, Verma M, Kumar D, 2016. MicroRNAs in glioblastoma multiforme pathogenesis and therapeutics. Cancer medicine, 5 (8), 1917–1946.

Shi L, Cheng Z, Zhang J, Li R, Zhao P, Fu Z, You Y, 2008. hsa-mir-181a and hsa-mir-181b function as tumor suppressors in human glioma cells. Brain research, 1236, 185–193.

Silantyev AS, Falzone L, Libra M, Gurina OI, Kardashova KS, Nikolouzakis TK, Nosyrev AE, Sutton CW, Mitsias PD, Tsatsakis A, 2019. Current and Future Trends on Diagnosis and Prognosis of Glioblastoma: From Molecular Biology to Proteomics. Cells, 8 (8).

Soeda A, Hara A, Kunisada T, Yoshimura S, Iwama T, Park DM, 2015. The evidence of glioblastoma heterogeneity. Scientific reports, 5, 7979.

Soeda A, Park M, Lee D, Mintz A, Androutsellis-Theotokis A, McKay RD, Engh J, Iwama T, Kunisada T, Kassam AB, Pollack IF, Park DM, 2009. Hypoxia promotes expansion of the CD133-positive glioma stem cells through activation of HIF-1alpha. Oncogene, 28 (45), 3949–3959.

Song H, Zhang Y, Liu N, Zhang D, Wan C, Zhao S, Kong Y, Yuan L, 2016. Let-7b inhibits the malignant behavior of glioma cells and glioma stem-like cells via downregulation of E2F2. Journal of physiology and biochemistry, 72 (4), 733–744.

Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJB, Janzer RC, Ludwin SK, Allgeier A, Fisher B, Belanger K, Hau P, Brandes AA, Gijtenbeek J, Marosi C, Vecht CJ, Mokhtari K, Wesseling P, Villa S, Eisenhauer E, Gorlia T, Weller M, Lacombe D, Cairncross JG, Mirimanoff R-O, 2009. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. The Lancet. Oncology, 10 (5), 459–466.

Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJB, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO, 2005. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. The New England journal of medicine, 352 (10), 987–996.

Swiatek-Machado K, Kaminska B, 2020. STAT Signaling in Glioma Cells. Advances in experimental medicine and biology, 1202, 203–222.

Tan AC, Ashley DM, López GY, Malinzak M, Friedman HS, Khasraw M, 2020. Management of glioblastoma: State of the art and future directions. CA: a cancer journal for clinicians, 70 (4), 299–312.

Turchinovich A, Weiz L, Langheinz A, Burwinkel B, 2011. Characterization of extracellular circulating microRNA. Nucleic acids research, 39 (16), 7223–7233.

Urabe F, Kosaka N, Ito K, Kimura T, Egawa S, Ochiya T, 2020. Extracellular vesicles as biomarkers and therapeutic targets for cancer. American journal of physiology. Cell physiology, 318 (1), C29-C39.

Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO, 2007. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nature cell biology, 9 (6), 654–659.

Valiulyte I, Pranckeviciene A, Bunevicius A, Tamasauskas A, Svitina H, Skrypkina I, Vaitkiene P, 2022. Associations of miR-181a with Health-Related Quality of Life, Cognitive Functioning, and Clinical Data of Patients with Different Grade Glioma Tumors. International Journal of Molecular Sciences, 23 (19), 11149.

van Niel G, D'Angelo G, Raposo G, 2018. Shedding light on the cell biology of extracellular vesicles. Nature reviews. Molecular cell biology, 19 (4), 213–228.

Verel-Yilmaz Y, Fernández JP, Schäfer A, Nevermann S, Cook L, Gercke N, Helmprobst F, Jaworek C, Pogge von Strandmann E, Pagenstecher A, Bartsch DK, Bartsch JW, Slater EP, 2021. Extracellular Vesicle-Based Detection of Pancreatic Cancer. Frontiers in cell and developmental biology, 9, 697939.

Vollmann-Zwerenz A, Leidgens V, Feliciello G, Klein CA, Hau P, 2020. Tumor Cell Invasion in Glioblastoma. International journal of molecular sciences, 21 (6).

Wang D, Wang X, Si M, Yang J, Sun S, Wu H, Cui S, Qu X, Yu X, 2020. Exosome-encapsulated miRNAs contribute to CXCL12/CXCR4-induced liver metastasis of colorectal cancer by enhancing M2 polarization of macrophages. Cancer letters, 474, 36–52.

Wang H, Tao T, Yan W, Feng Y, Wang Y, Cai J, You Y, Jiang T, Jiang C, 2015. Upregulation of miR-181s reverses mesenchymal transition by targeting KPNA4 in glioblastoma. Scientific reports, 5, 13072.

Wang J, Liu H, Tian L, Wang F, Han L, Zhang W, Bai Y-A, 2017a. miR-15b Inhibits the Progression of Glioblastoma Cells Through Targeting Insulin-like Growth Factor Receptor 1. Hormones & cancer, 8 (1), 49–57.

Wang P, Chen D, Ma H, Li Y, 2017b. LncRNA SNHG12 contributes to multidrug resistance through activating the MAPK/Slug pathway by sponging miR-181a in non-small cell lung cancer. Oncotarget, 8 (48), 84086–84101.

Wang X-F, Shi Z-M, Wang X-R, Cao L, Wang Y-Y, Zhang J-X, Yin Y, Luo H, Kang C-S, Liu N, Jiang T, You Y-P, 2012. MiR-181d acts as a tumor suppressor in glioma by targeting K-ras and Bcl-2. Journal of cancer research and clinical oncology, 138 (4), 573–584.

Wang X-R, Luo H, Li H-L, Cao L, Wang X-F, Yan W, Wang Y-Y, Zhang J-X, Jiang T, Kang C-S, Liu N, You Y-P, 2013. Overexpressed let-7a inhibits glioma cell malignancy by directly targeting K-ras, independently of PTEN. Neuro-oncology, 15 (11), 1491–1501.

Wei J, Marisetty A, Schrand B, Gabrusiewicz K, Hashimoto Y, Ott M, Grami Z, Kong L-Y, Ling X, Caruso H, Zhou S, Wang YA, Fuller GN, Huse J, Gilboa E, Kang N, Huang X, Verhaak R, Li S, Heimberger AB, 2019. Osteopontin mediates glioblastoma-associated macrophage infiltration and is a potential therapeutic target. The Journal of clinical investigation, 129 (1), 137–149.

Weller M, Felsberg J, Hartmann C, Berger H, Steinbach JP, Schramm J, Westphal M, Schackert G, Simon M, Tonn JC, Heese O, Krex D, Nikkhah G, Pietsch T, Wiestler O, Reifenberger G, Deimling A von, Loeffler M, 2009. Molecular predictors of progression-free and overall survival in patients with newly diagnosed glioblastoma: a prospective translational study of the German Glioma Network. Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 27 (34), 5743–5750.

Wen PY, Packer RJ, 2021. The 2021 WHO Classification of Tumors of the Central Nervous System: clinical implications. Neuro-oncology, 23 (8), 1215–1217.

Wen X, Li S, Guo M, Liao H, Chen Y, Kuang X, Liao X, Ma L, Li Q, 2020. miR-181a-5p inhibits the proliferation and invasion of drug-resistant glioblastoma cells by targeting F-box protein 11 expression. Oncology letters, 20 (5), 235.

Wu A, Wei J, Kong L-Y, Wang Y, Priebe W, Qiao W, Sawaya R, Heimberger AB, 2010. Glioma cancer stem cells induce immunosuppressive macrophages/microglia. Neuro-oncology, 12 (11), 1113–1125.

Wu Z, Guo J, Zhang Y, Liu J, Ma H, Tang Y, 2021. MiR-425-5p accelerated the proliferation, migration, and invasion of ovarian cancer cells via targeting AFF4. Journal of ovarian research, 14 (1), 138.

Wuebben EL, Mallanna SK, Cox JL, Rizzino A, 2012. Musashi2 is required for the self-renewal and pluripotency of embryonic stem cells. PloS one, 7 (4), e34827.

Xiao S, Zhu H, Luo J, Wu Z, Xie M, 2019. miR-425-5p is associated with poor prognosis in patients with breast cancer and promotes cancer cell progression by targeting PTEN. Oncology reports, 42 (6), 2550–2560.

Zarkoob H, Taube JH, Singh SK, Mani SA, Kohandel M, 2013. Investigating the link between molecular subtypes of glioblastoma, epithelial-mesenchymal transition, and CD133 cell surface protein. PloS one, 8 (5), e64169.

Zhai F, Chen X, He Q, Zhang H, Hu Y, Wang D, Liu S, Zhang Y, 2019. MicroRNA-181 inhibits glioblastoma cell growth by directly targeting CCL8. Oncology letters, 18 (2), 1922–1930.

Zhou J-S, Yang Z-S, Cheng S-Y, Yu J-H, Huang C-J, Feng Q, 2020. miRNA-425-5p enhances lung cancer growth via the PTEN/PI3K/AKT signaling axis. BMC pulmonary medicine, 20 (1), 223.

7. Publications

7.1 The Metalloprotease-Disintegrin ADAM8 alters the tumor suppressor miR-181a-5p expression profile in glioblastoma thereby contributing to its aggressiveness

frontiers in Oncology

ORIGINAL RESEARCH
published: 15 March 2022
doi: 10.3389/fonc.2022.826273

The Metalloprotease-Disintegrin ADAM8 Alters the Tumor Suppressor miR-181a-5p Expression Profile in Glioblastoma Thereby Contributing to Its Aggressiveness

Agnes Schäfer[1†], Lara Evers[1†], Lara Meier[1], Uwe Schlomann[1], Miriam H. A. Bopp[1,2], Gian-Luca Dreizner[1], Olivia Lassmann[1], Aaron Ben Bacha[1], Andreea-Cristina Benescu[1], Mirza Pojskic[1], Christian Preußer[3], Elke Pogge von Strandmann[3], Barbara Carl[1], Christopher Nimsky[1,2] and Jörg W. Bartsch[1,2]*

[1] Department of Neurosurgery, Philipps University Marburg, Marburg, Germany, [2] Marburg Center for Mind, Brain and Behavior (MCMBB), Marburg, Germany, [3] Core Facility Extracellular Vesicles, Philipps University of Marburg – Medical Faculty, Marburg, Germany

Glioblastoma (GBM) as the most common and aggressive brain tumor is characterized by genetic heterogeneity, invasiveness, radio-/chemoresistance, and occurrence of GBM stem-like cells. The metalloprotease-disintegrin ADAM8 is highly expressed in GBM tumor and immune cells and correlates with poor survival. In GBM, ADAM8 affects intracellular kinase signaling and increases expression levels of osteopontin/SPP1 and matrix metalloproteinase 9 (MMP9) by an unknown mechanism. Here we explored whether microRNA (miRNA) expression levels could be regulators of MMP9 expression in GBM cells expressing ADAM8. Initially, we identified several miRNAs as dysregulated in ADAM8-deficient U87 GBM cells. Among these, the tumor suppressor miR-181a-5p was significantly upregulated in ADAM8 knockout clones. By inhibiting kinase signaling, we found that ADAM8 downregulates expression of miR-181a-5p *via* activation of signal transducer and activator of transcription 3 (STAT3) and mitogen-activated protein kinase (MAPK) signaling suggesting an ADAM8-dependent silencing of miR-181a-5p. In turn, mimic miR-181a-5p transfection caused decreased cell proliferation and lower MMP9 expression in GBM cells. Furthermore, miR-181a-5p was detected in GBM cell-derived extracellular vesicles (EVs) as well as patient serum-derived EVs. We identified miR-181a-5p downregulating MMP9 expression *via* targeting the MAPK pathway. Analysis of patient tissue samples (n=22) revealed that in GBM, miR-181a-5p is strongly downregulated compared to *ADAM8* and *MMP9* mRNA expression, even in localized tumor areas. Taken together, we provide evidence for a functional axis involving ADAM8/miR-181a-5p/MAPK/MMP9 in GBM tumor cells.

Keywords: glioblastoma, tumor microenvironment, extracellular vesicles, miRNA, MR spectroscopy, ADAM8, miR-181a-5p, MMP9

OPEN ACCESS

Edited by:
Bozena Kaminska, Nencki Institute of Experimental Biology (PAS), Poland

Reviewed by:
Katarzyna Rolle, Institute of Bioorganic Chemistry (PAS), Poland
Francesca Peruzzi, Louisiana State University, United States

***Correspondence:**
Jörg W. Bartsch
jbartsch@med.uni-marburg.de

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Neuro-Oncology and Neurosurgical Oncology, a section of the journal Frontiers in Oncology

Received: *30 November 2021*
Accepted: *16 February 2022*
Published: *15 March 2022*

Citation:
Schäfer A, Evers L, Meier L, Schlomann U, Bopp MHA, Dreizner G-L, Lassmann O, Ben Bacha A, Benescu A-C, Pojskic M, Preußer C, von Strandmann EP, Carl B, Nimsky C and Bartsch JW (2022) The Metalloprotease-Disintegrin ADAM8 Alters the Tumor Suppressor miR-181a-5p Expression Profile in Glioblastoma Thereby Contributing to Its Aggressiveness. Front. Oncol. 12:826273. doi: 10.3389/fonc.2022.826273

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common malignant primary brain tumor in adults. Despite a standard multimodal therapeutic strategy combining maximum safe surgical resection and radio-/chemotherapy with temozolomide, the median survival remains low between 12 and 15 months (1). To improve the poor prognosis of GBM patients it is crucial to identify new therapeutic targets and their underlying dysregulated signaling pathways.

GBM is characterized as a highly invasive, heterogeneously composed, and rapidly growing tumor (2). At the molecular level, a disintegrin and metalloproteinases (ADAMs) mediate tumor cell adhesion and migration as well as intracellular signaling (3). One such proteolytically active family member is the metalloproteinase-disintegrin 8 (ADAM8), strongly associated with tumor aggressiveness, progression, and reduced survival in various cancers including breast cancer, pancreatic ductal adenocarcinoma (PDAC), and GBM (4–7). ADAM8, in particular, the cytoplasmic domain (CD) and the disintegrin/cysteine-rich domain (DC) can activate central signaling pathways in carcinogenesis. First, ADAM8 activates the mitogen-activated protein kinase (MAPK) signaling cascade, epidermal growth factor receptor (EGFR) independently (8, 9). Second, ADAM8 mediates angiogenesis by inducing the expression of osteopontin (*SPP1*) *via* STAT3 signaling (10). Moreover, ADAM8 interacts with integrin ß1 (ITGB1) and thereby activates its downstream targets focal adhesion kinase (FAK), and the PI3K/AKT pathway (9, 11). Interestingly, ADAM8 dependent activation of the MAPK pathway as well the PI3K/AKT pathway enhanced temozolomide-chemoresistance in GBM cell lines (12). Considering these diverse functions of ADAM8 in intracellular signaling, we and others hypothesized that ADAM8 mediates these functions through the regulation of microRNAs and indeed, initial evidence came from a study in MDA-MB-231 breast cancer cells showing that ADAM8 regulates expression levels of miR-720 (13).

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate protein expression on a post-transcriptional level by binding and thereby silencing their target messenger RNAs (mRNAs) (14). In most cases, miRNAs lead to translational repression or even degradation of their specific target mRNAs (15). Therefore, dysregulated miRNA expression profiles alter many critical pathways related to cancer progression (16). Consequently, in GBM, a large number of miRNAs are reported to be dysregulated (17, 18). In GBM, miR-181a-5p is downregulated and functions as a tumor suppressor miRNA that inhibits the translation of oncogenic proteins that are linked to tumor progression such as osteopontin (*SPP1*) (19–21). This type of sialoprotein is highly expressed in GBM and plays a key role in tumor-tumor microenvironment communication by attracting macrophages and mediating their immune response (22). Furthermore, miR-181a-5p regulates cell apoptosis and cell colony formation by targeting B-cell lymphoma 2 (BCL-2), so that high expression levels of miR-181a-5p can induce radiosensitivity of U87 GBM cells (23, 24). In addition, miR-181a-5p contains inhibitory binding sites to members of the MAPK family and its downstream targets, namely mitogen-activated protein kinase kinase 1 (MEK1), cAMP response element-binding protein 1 (CREB-1), and extracellular signal-regulated kinase 2 (ERK2) (25, 26). Given an important functional role in GBM, the signaling pathways regulating miR-181a-5p itself, however, remain unclear.

Matrix metalloproteinase 9 (MMP9), a zinc-dependent endopeptidase, plays a central role in the process of tumor cell migration, infiltration, and metastasis (27). Matrix metalloproteinases degrade extracellular matrix molecules and basement membrane components and thereby contribute to glioma progression (28). Consequently, MMP9 is upregulated in GBM compared to its expression in the normal brain parenchyma (29). Gliomas that display high MMP9 levels are associated with an aggressive course and are linked to reduced survival (30). Previous studies demonstrated that MMP9 expression can be elevated *via* MAPK-signaling (31, 32). ADAM8 and MMP9 levels are correlated in GBM tissue samples as well breast cancer-derived brain metastasis (8, 33). Whether MMP9 can be directly targeted by miR-181a-5p or indirectly *via* miR-181a-5p induced downregulation of the MAPK pathway has not been explored yet.

Cancer invasion is closely associated with the interaction of infiltrating tumor cells and the tumor microenvironment (TME) (34). As a means of communication, extracellular vesicles (EVs) are secreted by tumor cells as well as by cells of the TME. Their cargo contains lipids and proteins as well as nucleic acids including miRNAs (35). Because EVs modulate tumor growth, immune-escape, and tumor cell niche formation, they function as central regulators of the TME (34).

In the current work, we explored the mechanism by which ADAM8 modulates intracellular and extracellular signaling through the regulation of miR-181a-5p expression and uncovered *MMP9* as a miR-181a-5p dependent target gene in GBM.

MATERIAL AND METHODS

Patient Specimens

In accordance with the local ethics committee (Philipps University Marburg, medical faculty, file number 185/11), tumor tissue samples of GBM patients were obtained during surgical resection and serum specimens were collected one to three days prior and three to five days after surgical resection. Each patient gave written informed consent before resection. Tissue samples were shock frozen in liquid nitrogen and then stored at -80°C. Serum samples were centrifuged at 2,000 g for 10 min prior to storage at -80°C. All included tissue and serum samples were from primary, isocitrate dehydrogenase (IDH) wild-type GBM tumors, further patient information and histopathological characteristics are summarized in **Table 1**. In three cases, we analyzed the expression of miR-181a-5p in serum-derived EVs at the time of initial manifestation and tumor recurrence (Patient 9, 23, 24 in **Table 1**).

TABLE 1 | Clinical data on patient included tumor tissue samples showed isocitrate dehydrogenase (IDH) wild-type expression.

Number	Age at diagnosis (years)	Sex	Tumor localization	Type of resection	MGMT promoter methylation	EGFR vIII	Ki67-Li	Survival (days)
1	71	m	Septum pellucidum	Subtotal	Methylated	–	Up to 30%	114
2	65	m	Left parietal lobe	Subtotal	Not methylated	+	Up to 40%	119
3	77	w	Right frontal lobe	Subtotal	Methylated	–	Up to 20%	79
4	75	m	Right temporal and parietal lobe	Subtotal	Methylated	–	10%	476
5	63	w	Left frontal lobe	Subtotal	Methylated	–	Up to 30%	76
6	87	m	Right parietal and occipital lobe	Gross Total	Methylated	++	5%	135
7	78	w	Butterfly glioma, predominantly right frontal lobe	Subtotal	Not methylated	unknown	20%	63
8	66	m	Left frontal lobe	Subtotal	Methylated	–	25%	49
9*	66	m	Right occipital lobe	Gross total	Not methylated	+	20%	336
10	65	w	Left temporal lobe	Subtotal	Not methylated	–	Up to 15%	84
11	70	w	Left frontal lobe	Subtotal	Methylated	+	Up to 25%	278
12	61	m	Right temporal lobe	Gross total	Methylated	–	30%	626
13	64	w	Right frontal and temporal lobe	Gross total	Methylated	–	20%	930
14	65	m	Left temporal lobe	Subtotal	Methylated	–	30%	579
15	66	m	Right temporal lobe and right Insula	Subtotal	Methylated	–	Up to 20%	126
16	61	m	Left temporal lobe	Gross total	Not methylated	–	50%	398
17	57	w	Right frontal lobe	Gross total	Weakly methylated	+	20%	410
18	62	m	Right temporal and parietal lobe	Gross total	Not methylated	–	Up to 20%	457
19	56	m	Left temporal lobe	Gross total	Methylated	–	20%	578
20	69	m	Right parietal and occipital lobe	Gross total	Weakly methylated	–	Up to 50%	388
21	61	m	Right frontal lobe	Gross total	Weakly methylated	–	30%	94
22	76	m	Right frontal lobe	Gross total	Not methylated	–	30%	225
23*	76	f	Left parietal	Gross total	Methylated	–	20%	unknown
24*	54	m	Right frontal lobe	Gross total	Methylated	–	30%	450
25	74	f	Left parietal lobe	Gross total	Methylated	–	40%	unknown

Only initial manifested primary glioblastomas were included. Here, we show further parameters regarding the patient cohort including age at diagnosis, sex, survival in days, and type of surgical resection (gross total or subtotal). Furthermore, histopathological data such as methylation status of the O^6-methylguanine-DNA-methyltransferase (MGMT), Ki67-Labelling index (Ki67-Li), and expression of epidermal growth factor variant III (EGFRvIII) are presented here. Patients' 1 to 22 tissue samples were analyzed for miR-181a-5p, ADAM8, *and* MMP9 *mRNA expression (**Figures 5A–D**), matched samples (initial and recurrence GBM) from patients 9, 23, and 24 (*) were used for serum-EV separation and analysis (**Figures 5H–J**) and patient 25 was used for the analysis via MR-spectroscopy (**Figures 5E–G**).*

Cell Culture

Established GBM cell lines U87 and U251 were purchased from the American Type Culture Collection (ATCC) and cell lines G112 and G28 were obtained from the Westphal Lab (UKE Hamburg). All GBM cell lines were cultivated in Dulbecco's modified Eagle's medium (DMEM) high glucose (4.5 g/L) phenol red (Capricorn Scientific, Germany), supplemented with 10% fetal calf serum (FCS, S0615, Sigma, Germany), 1% penicillin/streptomycin (2321115, Gibco, US), 1% sodium pyruvate (NPY-B, Capricorn Scientific, Germany) and 1% non-essential amino acids (11140050, Gibco, US). Primary GBM cell lines and primary glioblastoma stem-like cells (GSCs) were obtained during surgical resection. The isolation and preparation process of GSCs and primary differentiated patient-derived GBM tumor cells were each described previously by our group (12, 36). GSC lines 2017/151, 2017/74, and 2016/240 were cultivated in DMEM/F12 (DMEM-12-A, Capricorn Scientific, Germany) and supplemented with 2% B27 supplement (117504044, Gibco, US), 1% amphotericin (152290026, Gibco, US), 0.5% HEPES (H0887, Sigma, Taufkirchen, Germany) and 0.1% Gentamycin (A2712, Biochrom, Germany). Moreover, a final concentration of 0.02 ng/µL EGF (100-18B, Peprotech, Germany) and bFGF (315-09, Peprotech, Germany) was added, and GSCs were cultivated in non-cell-culture-treated petri dishes. Primary differentiated GBM cell lines GBM98, GBM42, and GBM29 were cultivated in DMEM high glucose (4.5 g/L) without phenol red (Capricorn Scientific, Germany) supplemented with 10% FCS (S0615, Sigma, Germany), 1% penicillin/streptomycin (2321115, Gibco, US), 1 mM sodium pyruvate (NPY-B, Capricorn Scientific, Germany), 1% L-glutamine (200 mM) (25030-024, Gibco, US) and 1% non-essential amino acids (11140050, Gibco, US). All cell lines were cultured in a humidified atmosphere at 37°C under 5% CO_2.

Generation of Stable U87 CRISPR/Cas9 ADAM8 KO (KO) Clones

U87 cells were transfected with two different gRNAs using the CRISPR/Cas9 knockout/knockin kit from OriGene (#

KN213386) as described previously (37). Cell clones were selected by treatment with antibiotics (1 mg/ml puromycin). The ADAM8 knockout was confirmed through RT-qPCR, western blot, and ELISA analysis. U87 wild-type cells were used as control cells.

Transient Transfection to Induce an ADAM8 Rescue in U87 ADAM8 KO Cells

To rescue ADAM8 in U87 ADAM8 KO clones, cells were seeded in 6-well-plates at a density of 500,000 cells in 2 ml. After 24 h, the transfection was performed with either ADAM8 lacking the cytoplasmatic domain or the full-length ADAM8 using LTX Lipofectamine (Invitrogen) according to the manufacturer's instructions. Cells were harvested and analyzed by RT-qPCR and western blot after 48 h of transfection.

MiR-181a-5p Mimic Transfection

To transiently overexpress miR-181a-5p, U87 cells were seeded in 6-well-plates at a density of 400,000 cells in 2 ml and were transfected with 0.01 µM miR-181a-5p mimic (miScript, Qiagen) after 24 h. 0.01 µM ON-TARGET *plus* non-targeting Control Pool (Dharmacon, US) was used as control RNA. Transfection was performed utilizing Lipofectamine RNAimax (Invitrogen, UK) according to the manufacturer's instructions. After 24 h, the transfection was repeated. Transfected cells and their controls were harvested 48 h after the second transfection. To evaluate the success of transfection, miRNA expression was analyzed by RT-qPCR.

Inhibitors

Batimastat was used as a broad-spectrum MMP-inhibitor and was purchased from Tocris (Biotechne, Wiesbaden, Germany). As a specific ADAM8-inhibitor, BK-1361 (Peptide 2.0) was utilized and described by our group previously (9). WP´066 (Sigma Aldrich, US) was used as a JAK2/STAT3 inhibitor. Cells were seeded in a 6-well-format (500,000 cells in 2 ml) and harvested 16 h after treatment with inhibitors. The concentrations used are indicated in the graphs.

Separation of Extracellular Vesicles (EVs)

EVs were separated from cellular supernatants and GBM patients' serum samples *via* sequential ultracentrifugation. Cells were incubated with 30 ml DMEM supplemented with 1% L-glutamine (200 mM), 1% penicillin/streptomycin, 1 mM sodium pyruvate solution, and 1% nonessential amino acids for 48 h. Prior to EV separation, serum samples were diluted 1:3 with HBSS (Gibco™, Life Technologies, US) (500 µl serum diluted with 1 ml HBSS). The conditioned medium and the diluted serum sample were centrifuged first at 2,000 g for 10 min at RT and then at 10,000 g for 60 min at 4°C. After a subsequent filtration (0.2 µm filter), EVs were pelleted *via* high-speed centrifugation at 100,000 g for 90 min at 4°C using an Optima XPN-80 ultracentrifuge (Beckman Coulter, Germany). Next, the EV pellet was washed with HBSS at 100,000 g for 90 min at 4°C using the Optima MAX-XP (Beckman Coulter, Germany) ultracentrifuge with a TLA-55 fixed angle rotor. EVs were resuspended in 50 µl HBSS and stored at -80°C until further use. A 5 µl aliquot was sent to the FACS Core Facility, Marburg, for determining the size and concentration of the particles by usage of nano-flow cytometry (NanoFCM Co. Ltd., Nottingham, UK).

Real-Time Quantitative Polymerase Chain Reaction (qPCR)

Total RNA with an enriched fraction of miRNAs from tumor tissue samples and cellular pellets was isolated using the miRNeasy Tissue/Cells Advanced Mini Kit (217684, Qiagen, Germany) according to the manufacturer's instructions. To quantify the miRNA expression in cells, miRCURY LNA RT Kit (Cat. Number 339340, Qiagen, Germany) and miRCURY LNA SYBR® Green PCR Kit (Cat. Number 339345, Qiagen, Germany) were used according to manufacturer's instructions. YP00203_U6 snRNA miRCURY LNA PCR Assay (YP00203907, Qiagen, Germany) and miRCURY miRNA Assay hsa-181a-5p (YP00206081, Qiagen, Germany) was used for the quantification of relative miR-181a-5p expression. In the case of tissue samples (**Figure 5**), miScript II RT Kit (218161, Qiagen, Germany) and miScript SYBR Green PCR Kit (218073, Qiagen, Germany) were used according to the manufacturer's protocols. Here, Hs_RNU6-2_11 miScript Primer Assay (MS00033740, Qiagen, Germany) and Hs_miR-181a_2 miScript Primer Assay (MS00008827, Qiagen, Germany) were used. To assess gene expression on an mRNA level, RNA was reverse transcribed with RNA to cDNA EcoDry™ Premix (Takara Bio. Inc.). Quantitative real-time PCR was performed with iTaq™ Universal SYBR Green Supermix (Bio-rad Laboratories GmbH, US). QuantiTect Primer Assay (Qiagen) or forward and reverse primer were used in a total reaction volume of 20 µl. XS13 was used as a housekeeping gene. All PCR experiments were performed on the Applied Biosystems StepOnePlus Real-time PCR system (Thermo Fisher Scientific, US). Relative gene expression was calculated utilizing either the $2^{-\Delta Ct}$- or the $2^{-\Delta\Delta Ct}$ -method as indicated.

MiRNA PCR Array – Human Finder

A pathway-focused miRNA PCR Array/Human Finder (331221 miScript, MIHS-001ZC, Qiagen, Germany) was conducted according to the manufacturer's instructions. Data analysis was performed with the online miScript miRNA Data Analysis program from Qiagen using the $2^{-\Delta\Delta Ct}$-method. Results are presented in a heatmap.

Protein Extraction and Western Blot Analysis

Cells were washed with PBS (Sigma-Aldrich, US) and detached by cell scraping. Whole cell lysates were homogenized by an incubation for 30 min in RIPA buffer (50 mM HEPES pH 7.4; 150 mM NaCl; 1% (v/v) NP-40; 0.5% (w/v) Natriumdeoxycholate; 0.1% (w/v) SDS; 10 mM Phenantrolin; 10 mM EDTA; Pierce™ Protease Inhibitor Mini Tablets, EDTA-free, Thermo Fisher Scientific; Pierce™ Phosphatase Inhibitor Mini Tablets, Thermo Fisher Scientific). Protein samples or EVs in a concentration of $1.5x10^9$ particles were prepared in 5x Laemmli buffer [60 mM Tris-HCl pH

6.8; 2% (w/v) SDS; 10% (w/v) Glycerol; 5% (v/v) ß-Mercaptoethanol; 0.01% (w/v) Bromophenol-Blue] and 10x NuPAGE™ sample reducing reagent (Thermo Fisher Scientific, US) and denatured at 95°C for 5 min before SDS PAGE. For this, a 10% SDS polyacrylamide gel was used. Separated proteins were transferred on nitrocellulose membranes (A29591442, GE Healthcare Life science, Germany) followed by blocking in 5% (w/v) milk powder (MP) in TBST (50 mM Tris, pH 7.5; 150 mM NaCl; 0.1% (w/v) Tween-20) for 1 h. The detection of proteins was performed utilizing the following primary antibodies diluted as indicated in 5% MP in TBST: anti-ADAM8 (PA5-47047, Thermo Fisher Scientific, 1:1000), anti-MMP9 (IM09L, Calbiochem, 1:1,000), anti- β-Tubulin (NB600-936, Novus Biological, 1:2,000) anti-EGFR (4267, Cell Signaling, 1:1,000), anti-pEGFR (3777, Cell Signaling, 1:1,000), anti-MAPK (4696, Cell Signaling, 1:2,000), anti-pMAPK (4370, Cell Signaling, 1:2000), anti-CALNEXIN (2679, Cell Signaling, 1:1,000), anti-FLOTILLIN-1 (PA5-18053, Thermo Scientific, 1:2,000) anti-CD81 (sc166029, Santa Cruz, 1:500), anti-STAT3 (ab68153, Abcam, 1:5,000), anti-pSTAT3 (ab76315, Abcam, 1:5,000), anti-CREB-1 (H74) (sc-25785, Santa Cruz, 1:500 in 5% MP) and anti-pCREB-1(Ser133) (4276, Cell Signaling, 1:1000 in 5% BSA in TBST). Nitrocellulose membranes were incubated with primary antibodies at 4°C overnight. After washing three times with TBST, membranes were incubated with horseradish peroxidase (HRP) conjugated antibodies (Abcam, 1:5,000) for 1 h followed by a next washing step. Chemiluminescence detection was performed by adding Western Bright Sirius substrate (Advansta, US) and using the ChemiDoc MP Imaging System (Bio-rad Laboratories GmbH, US). Western blots were quantified using Image J (NIH, Maryland).

Enzyme-Linked Immunosorbent Assay (ELISA)

Soluble ADAM8 (DY1031, R&D Systems, UK) and soluble MMP9 (DY911, R&D Systems, UK) from cell culture supernatants were determined by Sandwich-ELISA method with DuoSet ELISA Kits. All ELISA experiments were performed according to the manufacturer's instructions.

Proliferation Assay

The proliferation and survival effects on U87 cells were determined using CellTiter-Glo 3D cell viability assay (G7571, Promega, Germany). Cells were seeded in triplicates on a 96-well plate. After 24 h, miR-181a-5p mimic was transfected according to section 2.5. After 48 h, 50 µl of CellTiter-Glo 3D Reagent was added to each well and mixed while shaking for 15 min. After an additional 15 min without shaking avoiding light, Luminescence was measured with a Microplate Reader luminometer (FLUOstar OPTIMA Microplate Reader, BMG Labtech, Germany).

Spectroscopy

A T2-weighted magnetic resonance (MR) tomography together with 1H-MR spectroscopy was performed on a 3T MR System (Trio, Siemens, Erlangen, Germany) for in detail analyses of tumor heterogeneity in patient 25. Thereby, a navigated extraction of tissue samples by co-registration of MR data and integration into the neuronavigation system (Curve Ceiling-Mounted, Brainlab, Munich, Germany) was enabled.

Statistical Analysis

Student's t-tests were applied for statistical analysis. For multiple comparisons, two-way ANOVA tests were used. A Wilcoxon-signed rank test and Pearson correlation were performed to determine differences or correlation in gene expression. Results were considered as not significant (ns) ($p > 0.05$), significant (*) ($p < 0.05$), highly significant (**) ($p < 0.01$), or very highly significant (***) ($p < 0.001$). Data from multiple replicates are presented as mean ± SD and statistical analyses were performed with GraphPad Prism (version 9.1.0) and Microsoft Excel.

RESULTS

ADAM8 Regulates Expression Levels of miR-181a-5p in GBM Cells

To determine potential ADAM8 correlated miRNAs, we generated stable ADAM8 knockout (KO) U87 cell clones using two guide RNAs (U87 gRNA cl. 1, U87 gRNA cl. 2) for the CRISPR/Cas9 homologous recombination method. U87 cells expressing high endogenous levels of ADAM8 were subjected to CRISPR/Cas9 induced genomic editing. After cell selection with puromycin, independent cell clones were grown and compared to U87 cells (in the following termed U87_CTRL) for morphological features and ADAM8 expression levels. From around 30 individual cell clones, two U87 gRNA clones were selected for further analyses (**Supplementary Figure 1**). Confirmation of successful ADAM8 knockout in these two U87 gRNA cell clones was provided by qPCR, Western Blot, and ELISA (**Figures 1A–C**). U87 gRNA cl. 1 and U87 gRNA cl. 2 (termed U87_KO1 and U87_KO2) showed a strong downregulation of *ADAM8* mRNA compared to U87_CTRL, $p < 0.001$ (**Figure 1A**). Western Blots confirmed successful ADAM8 knockout on the protein level (**Figure 1B**). In addition, ELISA measurements from cell supernatants revealed soluble ADAM8 levels below the detection limit in U87_KO clones compared to U87_CTRL ($p < 0.05$, **Figure 1C**). For two representative KO clones as well as a U87 control clone, a microRNA PCR Array (Human Finder) was screened. Differences in miRNA expression (given a ratio KO/CTRL) for both U87_KO clones are presented in a heatmap (**Figure 1D**) with green color representing upregulation of miRNA in U87_KO cells.

Several miRNAs were consistently upregulated in both KO clones and miR-181a-5p was selected for further investigations due to its reported regulation of osteopontin/SPP1 which also applies to ADAM8. Moreover, of all four miRNAs upregulated in U87 *ADAM8* KO cells, miR-181a-5p was the only one regulated after treatment of U87 wild-type cells with an ERK1/2 inhibitor indicating its influence in ADAM8-mediated signaling (**Supplementary Figure 2**). To further validate our miRNA screening, qPCR experiments were performed to detect miR-181a-5p expression in U87_KO and U87_CTRL cells. We

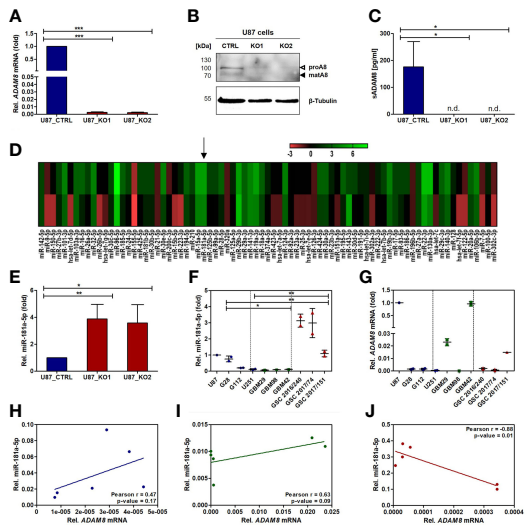

FIGURE 1 | Screening of two representative CRISPR/Cas9 ADAM8 (hA8) KO cell clones reveals an ADAM8-dependent regulation of miR-181a-5p. Confirmation of the CRISPR/Cas9 ADAM8 stable KO in U87 cells by qPCR **(A)**, Western Blot **(B)**, and ELISA **(C)**. **(D)** A RT-qPCR-based miRNA PCR Array (Human Finder, Qiagen) of U87 CRISPR/Cas9 ADAM8 KO cells enabling the analysis of a total of 84 miRNAs. The legend for the fold-changes in the heat map is given above representing the fold-change values ($2^{-\Delta\Delta CT}$) relative to U87_CTRL cells in both *ADAM8* KO clones. Note the variance in fold changes between the two KO clones **(E)** Confirmation of miR-181a-5p upregulation in U87_KO1 and U87_KO2 cells (p-value: 0.01 and 0.03). The expression of miR-181a-5p **(F)** and *ADAM8* **(G)** in GBM cell lines (G28, G112, U251), primary patient-derived cell lines (GBM29, 98, and 42), and GBM stem-like cell lines (GSCs) (GSC 2016/240, GSC 2017/74, GSC 2017/151) are shown as relative values to the expression in U87 cells. MiR-181a-5p is mostly expressed in GSCs. The patient-derived cell line GBM42 shows the highest ADAM8 expression. Pearson correlation analysis of miR-181a-5p and *ADAM8* in cell lines **(H)**, primary cell lines **(I)**, and primary GSCs **(J)** reveal a negative correlation only observed in GSCs (p-value: 0.01, Pearson r: -0.88). Results are given as mean +/- SD of two to three independent experiments. Unpaired two-tailed students *t-test* or two way ANOVA for multiple comparison **(F)** were applied to determine significance: *p < 0.05, **p < 0.01, ***p < 0.001. n.d., not detectable.

confirmed upregulation of miR-181a-5p in U87_KO1 and U87_KO2 compared to U87_CTRL cells, p < 0.05 and p < 0.01, respectively (**Figure 1E**).

Next, we analyzed the expression profiles of ADAM8 and miR-181a-5p in several GBM cell lines, including U87, U251, G112, G28, three primary patient-derived cell lines GBM42, GBM29, GBM98, and three patient-derived Glioblastoma stem-like cell lines (GSCs), 2016/240, 2017/151 and 2017/74 (**Figures 1F, G**). GSCs showed low ADAM8 mRNA and high miR-181a-5p expression levels. Primary GBM cell lines showed great variability in ADAM8 and miR-181a-5p expression with GBM42 with the highest ADAM8 levels. Interestingly, knocking ADAM8 down with siRNA showed elevated levels of miR-181a-5p in GBM42 (**Supplementary Figure 3**). Pearson correlation analyses revealed exclusively in the case of GSCs a clear negative correlation of ADAM8 and miR-181a-5p expression (**Figures 1H–J**). U87_CTRL cells as well as primary GBM42 cells showed the highest endogenous ADAM8 levels in qPCR experiments compared to all other cell lines and were selected for further experiments.

ADAM8 Regulates miR-181a-5p Expression *via* STAT3 and MAPK Signaling

To analyze the apparent ADAM8/miR-181a-5p dependence on the mechanistic level, we tested the contribution of either the metalloprotease activity or the functions of the non-proteolytic domains (DC/CD) of ADAM8 on miR-181a-5p expression. To address this, U87 cells were treated with either a broad-range metalloprotease inhibitor BB-94 (Batimastat) or with BK-1361, a selective ADAM8 inhibitor. While BB-94 did not affect miR-181a-5p expression, treatment with 10 μM and even 5 μM BK-1361 led to an increase in miR-181a-5p expression, p < 0.05 (**Figure 2A**) suggesting a contribution of the DC/CD domain on miR-181a-5p regulation by ADAM8. Moreover, we transiently re-expressed ADAM8 in U87_KO1 and analyzed the effect on miR-181a-5p expression. U87 gRNA_KO2 was transfected with either wild-type ADAM8 (hA8) or with an ADAM8 variant lacking the cytoplasmatic domain (Delta CD). Western Blots confirmed re-expression of ADAM8 variants (**Figure 2B**).

Re-expression of wild-type ADAM8 caused a downregulation of miR-181a-5p, p < 0.01 (**Figure 2B**). In contrast, cells expressing the ADAM8 delta CD variant showed no downregulation of miR-181a-5p (**Figure 2B**). These results indicate that the cytoplasmatic domain of ADAM8 triggers signaling cascades that lead to the downregulation of miR-181a-5p concomitant with a trend of increased pSTAT3 in cells transfected with wild-type ADAM8 (**Figure 2B**). Interestingly, this regulation only works in one direction, as changes in miR-181a-5p expression, i.e. by mimic transfection, do not affect expression levels of ADAM8 in U87 cells (**Figure 2C**). We explored the role of two downstream signaling pathways of ADAM8 CD, STAT3 signaling and MAPK signaling. For this purpose, U87 cells and primary GBM cells GBM42 were treated with either U0126 (MEK1/2 inhibitor) or WP1066 (STAT3 inhibitor). MEK1/2 inhibition caused an increase in miR-181a-5p expression in U87_CTRL cells (p < 0.05), and a tendency to increase in primary GBM42 cells (p-value: 0.052) (**Supplementary Figure 4**). More prominently, STAT3 inhibition by WP1066 was confirmed for both cell lines *via* western blot and resulted in increased expression levels of miR-181a-5p in both cell lines with p < 0.05 (**Figures 2D, E**).

MiR-181a-5p Regulates Cell Proliferation and MMP9 Expression

We further analyzed whether miR-181a-5p can affect the cell proliferation of GBM cells. Exemplified for U87_KO2, a decrease in cell proliferation was observed (p < 0.001, **Figure 3A**). This effect can be recapitulated when mimic miR-181a-5p was transfected into U87 cells (p < 0.01, **Figure 3B**). As an oncoprotein able to promote GBM cell proliferation, we analyzed *MMP9* expression in U87_KO2 and mimic transfected U87 cells (**Figures 3C, D**) (27). *MMP9* mRNA levels in U87_KO2 and mimic transfected cells are strongly downregulated as revealed by qPCR (p < 0.001, **Figure 3C**).

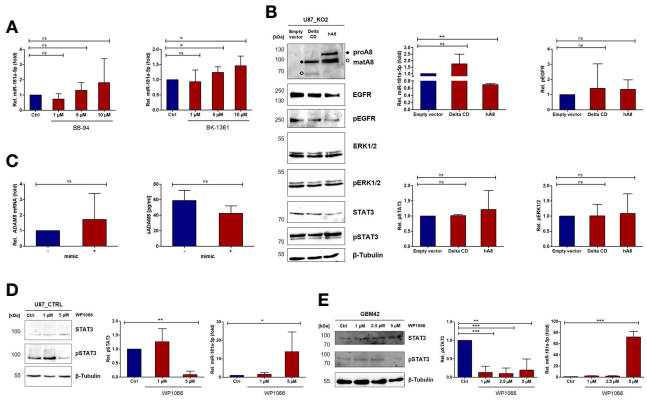

FIGURE 2 | ADAM8 regulates the expression of miR-181a-5p *via* JAK2/STAT3 signaling. **(A)** U87_CTRL cells were analyzed for miR-181a-5p expression by RT-qPCR after treatment with the broad-spectrum MMP inhibitor BB-94 (left) and the ADAM8 inhibitor BK-1361 (right). **(B)** One representative western blot of three independent experiments shows the rescue of either ADAM8 lacking the cytoplasmatic domain (Delta CD) or the full-length ADAM8 (hA8). The quantifications of pEGFR, pSTAT3, and pERK1/2 are depicted on the right side and were normalized to β-Tubulin and total-EGFR/β-Tubulin, total STAT3/β-Tubulin, or total ERK1/2/β-Tubulin. Also, RT-qPCR results show no differences in miR-181a-5p expression after the transfection of ADAM8 Delta CD but a downregulation with the full-length ADAM8 rescue (p-value: 0.002). **(C)** The expression of *ADAM8* mRNA (RT-qPCR, left) and secreted ADAM8 (ELISA, right, n=2) is not affected after miR-181a-5p mimic transfection. U87_CTRL cells **(D)** and patient-derived GBM42 cells **(E)** were treated with JAK2/STAT3 inhibitor WP1066 as indicated and analyzed *via* western blot and RT-qPCR. In **(D)**, qPCR results are shown as mean values +/- SD of four independent experiments and in **(E)**, results of miR-181a-5p are described as mean values of three technical replicates. Inhibition of JAK2/STAT3 increases miR-181a-5p expression (U87 p-value: 0.027; GBM42 p-value: 0.004). Results are shown as mean values +/- SD from three independent experiments if not otherwise stated. Unpaired two-tailed students *t-test* was applied to determine significance: ns, not significant, *p < 0.05, **p < 0.01, ***p < 0.001.

After mimic miR-181a-5p transfection of U87 cells, ELISA experiments revealed less soluble MMP9 levels in cellular supernatants ($p < 0.05$, **Figure 3D**). Comparable results were obtained for osteopontin (**Supplementary Figure 7**). Next, we explored whether miR-181a-5p dependent MMP9 downregulation is a result of direct miR-181-a/*MMP9* mRNA interaction. Three target prediction tools, miRDB, TargetScan, and TargetMiner, predicted no miR-181a-5p binding site. Also, bioinformatic analysis of the MMP9 3' UTR did not reveal a sufficiently long binding site for miR-181a-5p. Thus, we conclude that MMP9 is most likely indirectly regulated by miR-181a-5p. Indeed, literature research and the utilization of the target prediction tools miRDB and TargetScan revealed that miR-181a-5p directly targets three kinases of the MAPK pathway, CREB-1, MEK1, and ERK2 (**Supplementary Table 1**, 38, 39). To demonstrate that, transfection of U87_CTRL cells with a miR-181a-5p mimic was performed and revealed downregulation of pERK1/2 and p-CREB-1 in three independent Western Blot experiments, with $p < 0.01$ and $p < 0.001$, respectively (**Figure 3E**). Notably unphosphorylated levels of ERK1/2 and CREB-1 were not influenced by mimic transfection (**Figure 3E**). Thus, our results further support ERK2 and CREB-1 as downstream targets of miR-181a-5p.

EVs Derived From U87_KO Cells Are Associated With Higher miR-181a-5p Levels

Having demonstrated the intracellular effects of ADAM8 on miR-181a-5p and *MMP9* as a target gene, we further investigated whether EVs derived from cellular supernatants of U87_CTRL (CTRL_EVs), U87_KO1 (KO1_EVs), and U87_KO2 (KO2_EVs) are associated with miR-181a-5p expression. By Nanoflow Cytometry Measurement (NanoFCM), the size and concentration of EVs prepared from cellular supernatants were analyzed (**Figures 4A, B**). Western Blot experiments further confirmed the presence of EVs using FLOTILLIN-1 and CD81 as EV markers, CALNEXIN as a negative control, and ß-Tubulin as a predominant lysate marker (**Figure 4C**). MiR-181a-5p was detected in all three EV populations (CTRL_EVs, KO1_EVs, KO2_EVs) and consistent with our observation in U87_CTRL and U87_KO cells, KO1_EVs and KO2_EVs displayed higher miR-181a-5p levels than CTRL_EVs (**Figure 4D**). To ensure that more miR-181a-5p is packed in EVs with higher cellular expression, we separated EVs from ctrl and mimic transfected cells with a 28-fold enrichment of miR-181a-5p in EVs derived from mimic transfected cells (**Supplementary Figure 8**). Furthermore, we confirmed the uptake of KO2_EVs by U87_CTRL cells *via* immunofluorescent microscopy by incubating CFSE-stained KO2_EVs as well as CTRL_EVs with Hoechst-stained U87_CTRL cells (**Supplementary Figure 9A**). Western blot analysis showed downregulation of MMP9 in U87_CTRL cells incubated with both CTRL_EVs and KO2_EVs in comparison to the HBSS control but did not confirm a significant difference of MMP9 expression comparing cells incubated with KO2_EVs or CTRL-EVs (**Supplementary Figure 9B**). We treated U87_KO2 cells with either miR-181a-5p-mimics or a miR181a-5p inhibitor and incubated the corresponding EVs with U87_CTRL cells. An

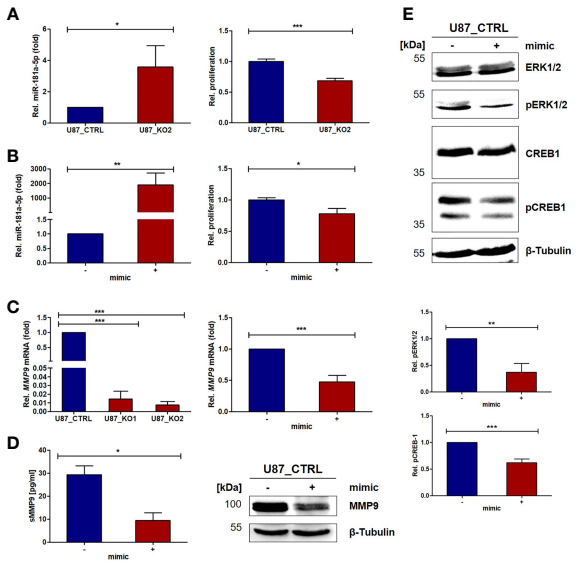

FIGURE 3 | ADAM8 affects MMP9 expression and proliferation *via* miR-181a-5p targeting ERK2 and CREB-1. **(A)** The upregulation of miR-181a-5p in U87_KO cells (RT-qPCR, n=3, left) causes inhibition of proliferation (CellTiter Glo, n=2, measurement after 48 h, right). **(B)** Overexpressing miR-181a-5p in U87_CTRL cells *via* transient transfection (RT-qPCR, n=3, left) causes inhibition of proliferation after 48 h (CellTiter Glo, n=3 technical replicates, right). **(C)** MMP9 is downregulated in U87_KO cells on mRNA (RT-qPCR, n=3, left). After miR-181a-5p mimic transfection, MMP9 is downregulated in U87 cells on the mRNA (RT-qPCR, n=3, p-value: 0.0009) **(C)** and on protein level (ELISA, n=2, p-value: 0.0004) analyzed by western blot **(D)**. **(E)** Analyses of kinase activation after miR-181a-5p mimic transfection for ERK1/2 (p-value: 0.003) and CREB-1 (p-value: 0.0007). Results are shown as mean values +/- SD of three independent experiments unless otherwise stated. Results are given in mean +/- SD. Unpaired one-tailed students *t* test was applied to determine significance: *p < 0.05, **p < 0.01, ***p < 0.001.

ELISA experiment revealed that incubation of inhibitor-treated EVs led to increased soluble MMP9 levels whilst incubation of miR-181a-5p-mimic treated EVs caused a decrease in soluble MMP9 (**Supplementary Figure 9C**).

Characterization of ADAM8, MMP9, and miR-181a-5p Expression in GBM Tumor Tissue Samples

RT-qPCR experiments were conducted on 22 tumor tissue samples from patients admitted to our clinical department to analyze the expression profiles of *ADAM8*, *MMP9*, and miR-181a-5p in GBM tissue. Further information on the patient cohort and histopathological data are listed in **Table 1**. For normalization of data (set to 1 in **Figures 5A–D**), we utilized tissue samples localized most remote from the tumor core. The majority of the examined tumor tissue samples showed downregulation of miR-181a-5p (**Figure 5C**). In contrast, mean *ADAM8* and *MMP9* expression levels were upregulated in the investigated tumor samples (**Figure 5A**). High *ADAM8* correlated with elevated *MMP9* expression levels, $p < 0.0001$ (**Figure 5D**). In the patient cohort, neither *ADAM8* mRNA levels nor *MMP9* mRNA levels correlated with miR-181a-5p expression, $p = 0.6$ and $p = 0.63$ respectively (**Figure 5D**). We then divided the patient cohort into subgroups, high *ADAM8* expression, and low *ADAM8* expression group, as well as high miR-181a-5p expression and low miR-181a-5p expression group (**Supplementary Figure 9**). *MMP9* expression was elevated in the high *ADAM8* group, $p = 0.01$ (**Figure 5B**). MiR-181a-5p expression was similar in the high *ADAM8* and low *ADAM8* groups (**Figure 5B**). *MMP9* expression was also similar in both miR-181a-5p subgroups (**Figure 5B**). To further investigate if this trend is due to the strong heterogeneity of the GBM tissue, we explored the connection between ADAM8, MMP9, and miR-181a-5p in a pilot experiment using MR-spectroscopy guided surgery at different locations in a GBM tumor tissue

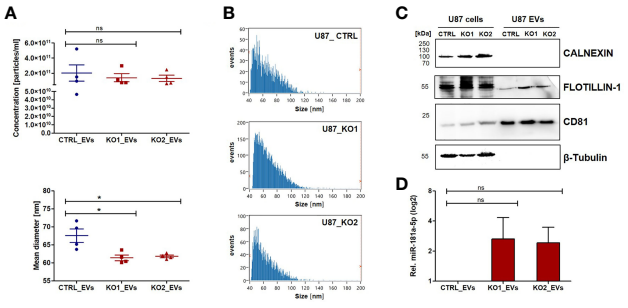

FIGURE 4 | ADAM8 affects miRNA181a-5p levels associated with EVs in U87 cells. **(A)** U87_CTRL and U87_KO derived EVs were characterized regarding their concentration (upper graph) and size (lower graph). Measurements were taken with the NanoFCM device and results are shown as mean values +/- SEM of four independent experiments. **(B)** Representative size distributions of U87_CTRL and U87_KO derived EVs (NanoFCM) are shown in bar graphs. **(C)** *Via* western blot, the presence of EVs was confirmed. CALNEXIN was used as negative control and FLOTILLIN-1 as well as CD81 as EV markers. **(D)** MiR-181a-5p is detectable in U87 derived EVs. RT-qPCR results show a non-significant tendency of miR-181a-5p upregulation in U87_ KO derived EVs. Mean values +/- SD of three independent experiments if not otherwise stated. Unpaired two-tailed students *t-test* was applied to determine significance: ns, not significant, *p < 0.05.

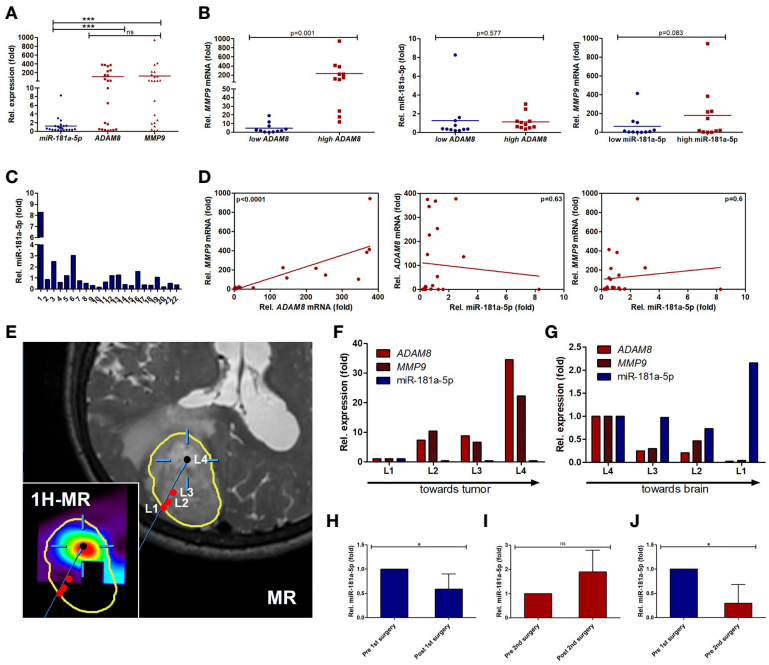

FIGURE 5 | Expression levels of *ADAM8* and *MMP9*, and miR-181a-5p in GBM tissue samples. **(A)** RT-qPCR results of GBM tissue samples (n=22, fold change normalized to 1) indicate a higher expression of *ADAM8* (p-value: 0.0009) and *MMP9* (p-value: 0.0002) than miR-181a-5p. **(B)** Dividing the RT-qPCR results and patient cohort into two groups (low/high *ADAM8* or low/high miR-181a-5p expression) reveals a correlation of *ADAM8* with *MMP9* (left graph, p-value: 0.001) but no correlation of miR-181a-5p with *ADAM8* (middle graph, p-value: 0.577) or *MMP9* (right graph, p-value: 0.083) expression. **(C)** RT-qPCR results for miR-181a-5p expression of each GBM tissue sample. **(D)** *ADAM8* and *MMP9* are correlated in GBM tissue samples ($p < 0.0001$, n=22), whereas the inverse correlations of *ADAM8* and miR-181a-5p and of miR-181a-5p and *MMP9* are non-significant (p-values: 0.63 and 0.6, respectively). **(E)** T2-weighted magnetic resonance (MR) image showing a left parietal GBM (segmented in yellow, patient 25) as well as the co-registered choline/N-acetylaspartate (NAA) maps derived from 1H-MR spectroscopy, integrated into the neuronavigation system for navigated extraction of tissue samples (L1: tumor border, L2/L3: tumor, L4: tumor, Cho/NAA hotspot) magnetic resonance (heatmap for choline metabolite). Corresponding molecular analyses are shown in **(F, G)** (patient 25). RT-qPCR results of *ADAM8* (red), *MMP9* (tiled red), and miR-181a-5p (blue) in different tissue locations normalized to either L1 **(F)** or L4 **(G)** describing the direction of surgery. **(H)** In a pilot study, three GBM patients (Patient 9, 23, 24) were analyzed for their serum-EV miR-181a-5p expression *via* RT-qPCR. The serum was collected before and after the first and second surgery. Interestingly, after first surgical resection miR-181a-5p is less expressed in serum-EVs (p-value: 0.042). **(I)** After second surgery, miR-181a-5p shows a slight increase in serum-EVs (p-value: 0.08). **(J)**, miR-181a-5p is less detectable in serum-EVs prior to the second surgery compared to pre-first surgery (p-value: 0.02; left graph). Results are shown in mean values +/- SD. Unpaired one-tailed students *t* test and Wilcoxon signed-rank test were applied to determine significance: ns, not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

sample of one selected patient. In the non-tumorous access tissue (L1), miR-181a-5p showed the highest expression whereas *ADAM8* and *MMP9* expression is at the lowest level (**Figures 5E–G**). Analysis of tumor edge (L2 and L3) and core tumor (L4) with strongly proliferating and vascularized zones revealed reversed expression patterns for *MMP9*, *ADAM8*, and miR-181a-5p (**Figures 5E–G**). Tumor locations in L3 and L4 were also confirmed by 1H-MR spectroscopy (**Supplementary Table 2**).

MiR-181a-5p Expression in Serum-Derived EVs From GBM Patients

In a further pilot study, serum specimens from three GBM patients were obtained before and after surgical resection. All three patients suffered from tumor recurrence and underwent surgical resection for a second time. In all cases, the highest miR-181a-5p expression levels were observed in serum samples prior to the first surgical resection (**Figure 5H**). After the first surgery, a reduction in miR-181a-5p levels was observed in post-surgery serum-derived EVs, $p < 0.05$ (5H). In contrast, after the second surgery, miR-181a-5p expression was slightly upregulated (**Figure 5I**). A comparison of primary manifested GBM and recurrent GBM revealed a decrease in miR-181a-5p expression in EVs, $p < 0.05$ (5K). These results suggest that miR-181a-5p could serve as a tumor marker, but needs to be sufficiently powered in further studies.

DISCUSSION

ADAM8 as a multidomain enzyme exhibits numerous tumor-supporting characteristics by promoting invasion, angiogenesis, and chemoresistance in GBM (10, 12). Due to these multiple functions, ADAM8 affects several intracellular pathways involving several important kinases and transcription factors

such as JAK2/STAT, AKT/PI3K, ERK1/2, and CREB-1 (8–12). Mechanistically, the ADAM8 metalloprotease domain cleaves extracellular membrane components while the cytoplasmatic domain activates crucial signaling cascades in carcinogenesis (4). Thereby, ADAM8 induces the expression of several oncoproteins including MMP9 and *SPP1*/osteopontin (8, 10). Previously, we demonstrated that ADAM8-dependent MMP9 expression is mediated *via* the MAPK pathway and resulted in a strong correlation of ADAM8 and MMP9 in breast cancer-derived brain metastasis (8). By characterizing the expression profile of ADAM8 and MMP9 in GBM tissue samples, we confirmed these observations for GBM. To dissect the effects of ADAM8 on oncoproteins mechanistically, we hypothesized that ADAM8 could alter the expression levels of distinct miRNAs such as miR-720, as previously shown for breast cancer cells (13). Generation of stable ADAM8 KO clones with subsequent miRNA screening revealed that the tumor suppressor miRNA miR-181a-5p shows a significantly higher expression in GBM cells deficient in ADAM8. Since high ADAM8 levels are correlated with GBM progression, a downregulation of miRNA181a-5p would be expected. Indeed, a recent study linked the poor prognosis of GBM patients with low miRNA181a-5p expression levels (40). Together, these findings qualified miRNA181a-5p as a candidate for a detailed molecular analysis, as presented here. Transient re-expression of ADAM8 in U87_KO cells resulted in downregulation of miR-181a-5p, suggesting that ADAM8 actively suppresses the expression of miR-181a-5p. Downregulation of miR-181a-5p by ADAM8 is dependent on the presence of the cytoplasmatic domain. In GBM, miR-181a-5p acts as a tumor suppressor miRNA by reducing invasiveness and enhancing radio- and chemosensitivity (23, 41). We confirmed that overexpression of miR-181a-5p led to reduced proliferation rates in U87 cells. Moreover, a similar effect on cell proliferation was observed in ADAM8 deficient GBM cells. It was shown that miR-181a-5p suppresses cell colony formation and tumor growth, and regulates apoptosis by targeting BCL-2 (23, 41). It is interesting to note that GSCs express relatively high levels of miRNA181a-5p compared to differentiated GBM cells, which could be instrumental in regulating proliferation and cell survival of this particular cell type. We have evidence that ADAM8 and, negatively correlated, miRNA181a-5p levels change in GSCs under conditions favoring differentiation of GSCs (Schäfer, unpublished data). However, the mechanisms that lead to miR-181a-5p downregulation in GBM remained elusive until now. As ADAM8 is a membrane-anchored protein, we concluded that ADAM8 downregulates the expression of miR-181a-5p by downstream signaling and activation of transcription factors. Indeed, our results revealed that miR-181a-5p can be downregulated by the activation of STAT3 and MAPK pathways. Conversely, miRNA181a-5p can regulate either total STAT3 levels in U87 cells and, notably, affect levels of p-STAT3 in the primary GBM cell line GBM42, indicating an unknown mechanism of kinase regulation by miRNA, similar to an observation made for phospho-AKT and p-ERK in a previous study in glioma (42) Previously, the importance of STAT3 signaling in GBM has been demonstrated in numerous studies whilst our group showed that ADAM8 dependent activation of STAT3 signaling led to increased angiogenesis by upregulation of osteopontin (10, 43, 44, reviewed in 45). In agreement with these findings, the 3'UTR of *SPP1*/osteopontin contains a binding site for miR-181a-5p and can be downregulated upon miR-181a-5p overexpression (19). All these results support the existence of a possible ADAM8/STAT3/miR-181a/osteopontin axis in GBM (**Figure 6** left). In addition, increased activation of the MAPK pathway is observed in numerous malignant tumors and leads to uncontrolled cell growth and mitosis (46). One of the best-known activators of the MAPK signaling pathway is the EGFR. Frequently, primary GBM tumors display a constitutively active variant, EGFRvIII (47). Apart from EGFR dependent MAPK activation, ADAM8 can activate the MAPK pathway EGFR independently (9). Interestingly, two kinases of the MAPK pathway, ERK2, and MEK1 as well as the downstream transcription factor CREB-1 are known to contain binding sites for miR-181a-5p (25, 26). In our experiments, phosphorylated and thus activated pCREB and pERK1/2 were downregulated in U87 cells transfected with miR-181a-5p mimics. We did not observe any effects on unphosphorylated CREB-1 and ERK1/2 as well as on MEK1/2 expression. Since miRNAs are post-transcriptional regulators of protein expression, we do not fully understand these results, but a TargetScan search revealed that CRBL2, a protein regulating phosphorylation of CREB1 is directly regulated by miR181a-5p, adding one more level of complexity to the network we have described here. A study by Fu et al. showed that CREB-1 suppresses miR-181a-5p transcription by directly binding to its promoter region (48). Thus, the interaction of miR-181a-5p and the MAPK pathway may constitute a regulatory loop that requires further investigation. Furthermore, we can postulate that our results describing the regulation of miR-181a-5p by ADAM8 are not restricted to the role of ADAM8 in GBM, as all other tumor cell lines that we investigated so far such as the triple-negative breast cancer cell line MDA-MB-231 and the PDAC cell line Panc89 show elevated levels of miR-181a-5p upon ADAM8 deficiency (unpublished observations). In accordance, MDA-MB-231 were among those cell lines that showed a strong correlation between ADAM8 and MMP9 expression in our previous study on breast cancer-derived brain metastases (8).

MMP9 plays a central role in tumor progression, especially for cell proliferation and invasion (27). Moreover, MMP9 expression is a prognostic factor in GBM and negatively correlated with patient survival (30). Thus, exploring the miR-181a-5p dependent *MMP9* downregulation was particularly interesting. U87 cells overexpressing miR-181a-5p exhibited decreased MMP9 levels. This was observed in U87 ADAM8 knockout cells as well as U87_CTRL cells incubated with miR-181a-5p mimics. To further establish whether *MMP9* mRNA contains a binding site for miR-181a-5p, we utilized target prediction tools and analyzed the mRNA sequence of MMP9. However, this analysis revealed that the *MMP9* mRNA does not contain an authentic binding site for miR-181a-5p.

Consequently, we concluded that miR-181a-5p can indirectly downregulate MMP9 expression by silencing the MAPK cascade (**Figure 6** right). This conclusion is supported by data showing that ERK1/2 inhibition led to decreased MMP9 levels in U87 and GBM42 cells (**Supplementary Figure 5**).

All these results demonstrate intracellular regulatory mechanisms of ADAM8/miR-181a-5p signaling so far. Cell-cell communication in the tumor microenvironment is essential for shaping either an immunosuppressive or a tumor-supportive microenvironment (34). As one mode of cell-cell communication, tumor cells release EVs. These heterogeneous nanoparticles contain a great variety of different molecules including miRNAs (35). Clinically, EVs received increasing attention, as their function as novel diagnostic and prognostic biomarkers is discussed (49). In our study, we analyzed the miR-181a-5p expression in U87 cells and serum-derived EVs. We recapitulated the higher abundance of miR-181a-5p in EVs from ADAM8 KO cells. Concerning patient sera, miR-181-5p expression in EVs dropped after the first surgical tumor resection. Moreover, miR-181a-5p expression was downregulated in serum-derived EVs from recurrent GBM. These results suggest that miR-181a-5p is further downregulated along with tumor progression. However, additional analyses must be carried out in a larger patient cohort to support this conclusion. The uptake of EVs can alter the behavior of recipient cells (50). Therefore, EVs might also be utilized as therapeutic vehicles (51). In our experiments, miR-181a-5p enriched vesicles were taken up by naive U87 cells demonstrating a role for ADAM8 in the tumor microenvironment. It remains to be determined if GBM resident immune cells such as macrophages that constitutively express ADAM8 could release EVs that might fail to suppress MMP9 expression in target cells, in conjunction with the possible tumor-promoting role of ADAM8 in macrophages (33).

Due to limited therapeutic options as well as the absence of early diagnostic biomarkers, GBM remains challenging as an incurable disease with a grim prognosis. Therefore, the identification of potential biomarkers as well as new therapeutic targets is of high importance. In summary, we identified that ADAM8 downregulates miR-181a-5p by activation of STAT3 and MAPK signaling. Considering that miR-181a-5p is a tumor suppressor miRNA in GBM, ADAM8 dependent silencing of miR-181a-5p could further contribute to

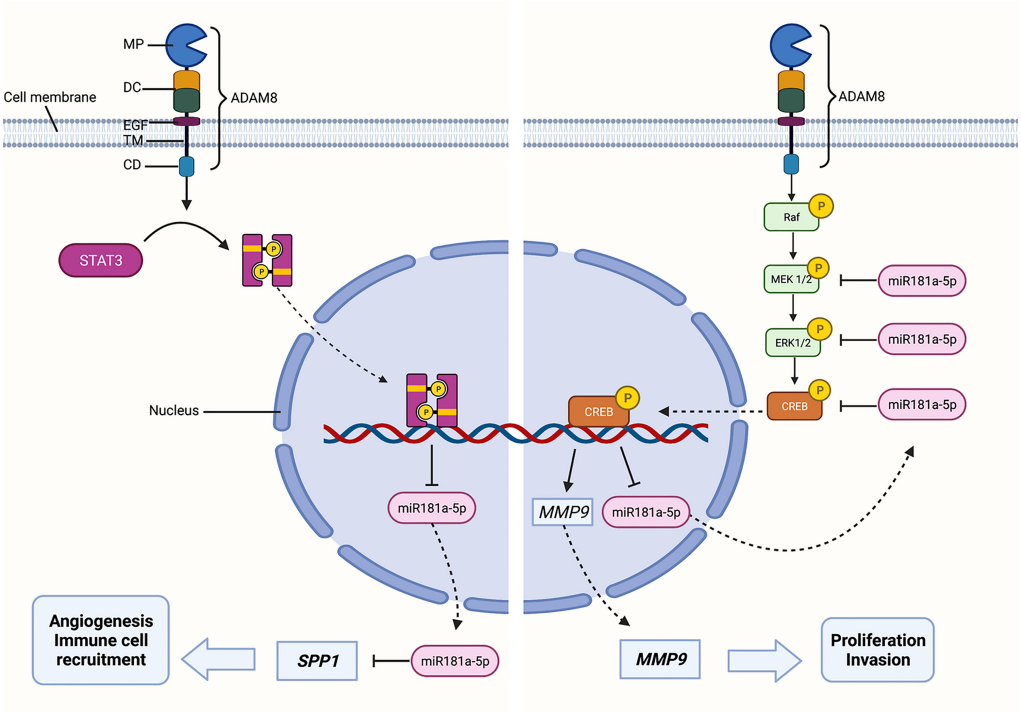

FIGURE 6 | Sketch of ADAM8-dependent effects caused by regulation of miR-181a-5p in GBM cells. ADAM8 with homologous domains including the metalloprotease domain (MP), the disintegrin/cysteine-rich domain (DC), the EGF-like domain (EGF), the transmembrane (TM), and the cytoplasmic domain (CD). ADAM8 activates intracellular signaling cascades by STAT3 and MAPK in the presence of the cytoplasmic domain. ADAM8/STAT3/miR-181a/*SPP1* axis: ADAM8 dependent STAT3 activation downregulates miR-181a-5p, as miR-181a-5p targets *SPP1*, disinhibition of *SPP1* leads to several tumor progressing effects such as induction of angiogenesis and enhanced immune cell recruitment. ADAM8/MAPK/MMP9 axis: ADAM8 activates the MAPK pathway, the transcription factor CREB-1 induces MMP9 transcription and inhibits miR-181a-5p transcription. MMP9 promotes tumor cell proliferation and invasion. By targeting CREB-1, ERK2, and MEK1, miR-181a-5p downregulates MMP9 expression most likely by an indirect mechanism. Created with BioRender.com.

tumor progression. We showed that overexpression of miR-181a-5p decreased cell proliferation and suppressed MMP9 expression by downregulation of the MAPK pathway Moreover, the presence of miR-181a-5p in clinical samples and EVs isolated from cellular supernatants as well as patient sera justifies further studies to reveal a potential role of miR-181a-5p in GBM diagnosis and progression.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Local Ethics Committee (Philipps University Marburg, medical faculty, file number 185/11). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JWB, ES, BC, CN, and MB conceived this study. AS, LE, LM, US, GLD, ABB, OL, and CP performed experiments and evaluated the data. ACB, MP, CN, and BC provided resources and clinical data. AS, LE, and JWB wrote the manuscript draft. MB, CN, and MP reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Work was supported in the framework of ERANET PerMed joint call 2018, project PerProGlio by the Federal Ministry for Education and Research (BMBF), grant number 01KU1915B to JWB, AS and by the Deutsche Forschungsgemeinschaft (DFG) grant BA1606/3-1 to US and JWB, and GRK 2573/1 to ES, and by a Research Grant from the University Medical Center Giessen and Marburg (UKGM). Open Access was kindly supported by the University of Marburg and the DFG.

ACKNOWLEDGMENTS

The authors thank Susanne Stei for her expert technical assistance. We also thank Dr. Miriam Frech (Clinic for Hematology and Oncology, Marburg) for her kind support with antibodies.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.826273/full#supplementary-material

REFERENCES

1. Alifieris C, Trafalis DT. Glioblastoma Multiforme: Pathogenesis and Treatment. *Pharmacol Ther* (2015) 152:63–82. doi: 10.1016/j.pharmthera.2015.05.005
2. Vollmann-Zwerenz A, Leidgens V, Feliciello G, Klein CA, Hau P. Tumor Cell Invasion in Glioblastoma. *Int J Mol Sci* (2020) 21(6):1932. doi: 10.3390/ijms21061932
3. Murphy G. The ADAMs: Signalling Scissors in the Tumour Microenvironment. *Nat Rev Cancer* (2008) 8(12):929–41. doi: 10.1038/nrc2459
4. Conrad C, Benzel J, Dorzweiler K, Cook L, Schlomann U, Zarbock A, et al. ADAM8 in Invasive Cancers: Links to Tumor Progression, Metastasis, and Chemoresistance. *Clin Sci (Lond)* (2019) 133(1):83–99. doi: 10.1042/CS20180906
5. He S, Ding L, Cao Y, Li G, Deng J, Tu Y, et al. Overexpression of a Disintegrin and Metalloprotease 8 in Human Gliomas is Implicated in Tumor Progression and Prognosis. *Med Oncol* (2012) 29(3):2032–7. doi: 10.1007/s12032-011-0084-9
6. Romagnoli M, Mineva ND, Polmear M, Conrad C, Srinivasan S, Loussouarn D, et al. ADAM8 Expression in Invasive Breast Cancer Promotes Tumor Dissemination and Metastasis. *EMBO Mol Med* (2014) 6(2):278–94. doi: 10.1002/emmm.20130337
7. Valkovskaya N, Kayed H, Felix K, Hartmann D, Giese NA, Osinsky SP, et al. ADAM8 Expression is Associated With Increased Invasiveness and Reduced Patient Survival in Pancreatic Cancer. *J Cell Mol Med* (2007) 11(5):1162–74. doi: 10.1111/j.1582-4934.2007.00082.x
8. Conrad C, Götte M, Schlomann U, Roessler M, Pagenstecher A, Anderson P, et al. ADAM8 Expression in Breast Cancer Derived Brain Metastases: Functional Implications on MMP-9 Expression and Transendothelial Migration in Breast Cancer Cells. *Int J Cancer* (2018) 142(4):779–91. doi: 10.1002/ijc.31090
9. Schlomann U, Koller G, Conrad C, Ferdous T, Golfi P, Garcia AM, et al. ADAM8 as a Drug Target in Pancreatic Cancer. *Nat Commun* (2015) 6:6175. doi: 10.1038/ncomms7175
10. Li Y, Guo S, Zhao K, Conrad C, Driescher C, Rothbart V, et al. ADAM8 Affects Glioblastoma Progression by Regulating Osteopontin-Mediated Angiogenesis. *Biol Chem* (2021) 402(2):195–206. doi: 10.1515/hsz-2020-0184
11. Awan T, Babendreyer A, Mahmood Alvi A, Düsterhöft S, Lambertz D, Bartsch JW, et al. Expression Levels of the Metalloproteinase ADAM8 Critically Regulate Proliferation, Migration and Malignant Signalling Events in Hepatoma Cells. *J Cell Mol Med* (2021) 25(4):1982–99. doi: 10.1111/jcmm.16015
12. Dong F, Eibach M, Bartsch JW, Dolga AM, Schlomann U, Conrad C, et al. The Metalloprotease-Disintegrin ADAM8 Contributes to Temozolomide Chemoresistance and Enhanced Invasiveness of Human Glioblastoma Cells. *Neuro Oncol* (2015) 17(11):1474–85. doi: 10.1093/neuonc/nov042
13. Das SG, Romagnoli M, Mineva ND, Barillé-Nion S, Jézéquel P, Campone M, et al. miR-720 is a Downstream Target of an ADAM8-Induced ERK Signaling Cascade That Promotes the Migratory and Invasive Phenotype of Triple-Negative Breast Cancer Cells. *Breast Cancer Res* (2016) 18(1):40. doi: 10.1186/s13058-016-0699-z
14. O'Brien J, Hayder H, Zayed Y, Peng C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front Endocrinol (Lausanne)* (2018) 9:402:402. doi: 10.3389/fendo.2018.00402
15. Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA Translation and Stability by microRNAs. *Annu Rev Biochem* (2010) 79:351–79. doi: 10.1146/annurev-biochem-060308-103103
16. Ali Syeda Z, Langden SSS, Munkhzul C, Lee M, Song SJ. Regulatory Mechanism of MicroRNA Expression in Cancer. *Int J Mol Sci* (2020) 21 (5):1723. doi: 10.3390/ijms21051723

17. Møller HG, Rasmussen AP, Andersen HH, Johnsen KB, Henriksen M, Duroux M. A Systematic Review of microRNA in Glioblastoma Multiforme: Micro-Modulators in the Mesenchymal Mode of Migration and Invasion. *Mol Neurobiol* (2013) 47(1):131–44. doi: 10.1007/s12035-012-8349-7
18. Shea A, Harish V, Afzal Z, Chijioke J, Kedir H, Dusmatova S, et al. MicroRNAs in Glioblastoma Multiforme Pathogenesis and Therapeutics. *Cancer Med* (2016) 5(8):1917–46. doi: 10.1002/cam4.775
19. Marisetty A, Wei J, Kong LY, Ott M, Fang D, Sabbagh A, et al. MiR-181 Family Modulates Osteopontin in Glioblastoma Multiforme. *Cancers (Basel)* (2020) 12(12):3813. doi: 10.3390/cancers1212381
20. Ciafrè SA, Galardi S, Mangiola A, Ferracin M, Liu CG, Sabatino G, et al. Extensive Modulation of a Set of microRNAs in Primary Glioblastoma. *Biochem Biophys Res Commun* (2005) 334(4):1351–8. doi: 10.1016/j.bbrc.2005.07.030
21. Wang H, Tao T, Yan W, Feng Y, Wang Y, Cai J, et al. Upregulation of miR-181s Reverses Mesenchymal Transition by Targeting KPNA4 in Glioblastoma. *Sci Rep* (2015) 5:13072. doi: 10.1038/srep13072
22. Wei J, Marisetty A, Schrand B, Gabrusiewicz K, Hashimoto Y, Ott M, et al. Osteopontin Mediates Glioblastoma-Associated Macrophage Infiltration and is a Potential Therapeutic Target. *J Clin Invest* (2019) 129(1):137–49. doi: 10.1172/jci121266
23. Chen G, Zhu W, Shi D, Lv L, Zhang C, Liu P, et al. MicroRNA-181a Sensitizes Human Malignant Glioma U87MG Cells to Radiation by Targeting Bcl-2. *Oncol Rep* (2010) 23(4):997–1003. doi: 10.3892/or_00000725
24. Shi L, Cheng Z, Zhang J, Li R, Zhao P, Fu Z, et al. Hsa-Mir-181a and Hsa-Mir-181b Function as Tumor Suppressors in Human Glioma Cells. *Brain Res* (2008) 1236:185–93. doi: 10.1016/j.brainres.2008.07.085
25. Wang P, Chen D, Ma H, Li Y. LncRNA SNHG12 Contributes to Multidrug Resistance Through Activating the MAPK/Slug Pathway by Sponging miR-181a in non-Small Cell Lung Cancer. *Oncotarget* (2017) 8(48):84086–101. doi: 10.18632/oncotarget.20475
26. Liu Y, Zhao Z, Yang F, Gao Y, Song J, Wan Y. microRNA-181a is Involved in Insulin-Like Growth Factor-1-Mediated Regulation of the Transcription Factor CREB1. *J Neurochem* (2013) 126(6):771–80. doi: 10.1111/jnc.12370
27. Huang H. Matrix Metalloproteinase-9 (MMP-9) as a Cancer Biomarker and MMP-9 Biosensors: Recent Advances. *Sensors (Basel)* (2018) 18(10):3249. doi: 10.3390/s18103249
28. Hagemann C, Anacker J, Ernestus RI, Vince GH. A Complete Compilation of Matrix Metalloproteinase Expression in Human Malignant Gliomas. *World J Clin Oncol* (2012) 3(5):67–79. doi: 10.5306/wjco.v3.i5.67
29. Musumeci G, Magro G, Cardile V, Coco M, Marzagalli R, Castrogiovanni P, et al. Characterization of Matrix Metalloproteinase-2 and -9, ADAM-10 and N-Cadherin Expression in Human Glioblastoma Multiforme. *Cell Tissue Res* (2015) 362(1):45–60. doi: 10.1007/s00441-015-2197-5
30. Li Q, Chen B, Cai J, Sun Y, Wang G, Li Y, et al. Comparative Analysis of Matrix Metalloproteinase Family Members Reveals That MMP9 Predicts Survival and Response to Temozolomide in Patients With Primary Glioblastoma. *PloS One* (2016) 11(3):e0151815. doi: 10.1371/journal.pone.0151815
31. Das G, Shiras A, Shanmuganandam K, Shastry P. Rictor Regulates MMP-9 Activity and Invasion Through Raf-1-MEK-ERK Signaling Pathway in Glioma Cells. *Mol Carcinog* (2011) 50(6):412–23. doi: 10.1002/mc.20723
32. Lakka SS, Jasti SL, Gondi C, Boyd D, Chandrasekar N, Dinh DH, et al. Downregulation of MMP-9 in ERK-Mutated Stable Transfectants Inhibits Glioma Invasion *In Vitro*. *Oncogene* (2002) 21(36):5601–8. doi: 10.1038/sj.onc.1205646
33. Gjorgjevski M, Hannen R, Carl B, Li Y, Landmann E, Buchholz M, et al. Molecular Profiling of the Tumor Microenvironment in Glioblastoma Patients: Correlation of Microglia/Macrophage Polarization State With Metalloprotease Expression Profiles and Survival. *Biosci Rep* (2019) 39(6):BSR20182361. doi: 10.1042/bsr20182361
34. Cavallari C, Camussi G, Brizzi MF. Extracellular Vesicles in the Tumour Microenvironment: Eclectic Supervisors. *Int J Mol Sci* (2020) 21(18):6768. doi: 10.3390/ijms21186768
35. van Niel G, D'Angelo G, Raposo G. Shedding Light on the Cell Biology of Extracellular Vesicles. *Nat Rev Mol Cell Biol* (2018) 19(4):213–28. doi: 10.1038/nrm.2017.125
36. Hannen R, Selmansberger M, Hauswald M, Pagenstecher A, Nist A, Stiewe T, et al. Comparative Transcriptomic Analysis of Temozolomide Resistant Primary GBM Stem-Like Cells and Recurrent GBM Identifies Up-Regulation of the Carbonic Anhydrase CA2 Gene as Resistance Factor. *Cancers (Basel)* (2019) 11(7):921. doi: 10.3390/cancers11070921
37. Scharfenberg F, Helbig A, Sammel M, Benzel J, Schlomann U, Peters F, et al. Degradome of Soluble ADAM10 and ADAM17 Metalloproteases. *Cell Mol Life Sci* (2020) 77(2):331–50. doi: 10.1007/s00018-019-03184-4
38. McGeary SE, Lin KS, Shi CY, Pham TM, Bisaria N, Kelley GM, et al. The Biochemical Basis of microRNA Targeting Efficacy. *Science* (2019) 366(6472):eaav1741. doi: 10.1126/science.aav1741
39. Chen Y, Wang X. miRDB: An Online Database for Prediction of Functional microRNA Targets. *Nucleic Acids Res* (2020) 48(D1):D127–31. doi: 10.1093/nar/gkz757
40. Huang SX, Zhao ZY, Weng GH, He XY, Wu CJ, Fu CY, et al. The Correlation of microRNA-181a and Target Genes With Poor Prognosis of Glioblastoma Patients. *Int J Oncol* (2016) 49(1):217–24. doi: 10.3892/ijo.2016.3511
41. Wen X, Li S, Guo M, Liao H, Chen Y, Kuang X, et al. miR-181a-5p Inhibits the Proliferation and Invasion of Drug-Resistant Glioblastoma Cells by Targeting F-Box Protein 11 Expression. *Oncol Lett* (2020) 20(5):235. doi: 10.3892/ol.2020.12098
42. Wang XF, Shi ZM, Wang XR, Cao L, Wang YY, Zhang JX, et al. MiR-181d Acts as a Tumor Suppressor in Glioma by Targeting K-Ras and Bcl-2. *J Cancer Res Clin Oncol* (2012) 138(4):573–84. doi: 10.1007/s00432-011-1114-x
43. Priester M, Copanaki E, Vafaizadeh V, Hensel S, Bernreuther C, Glatzel M, et al. STAT3 Silencing Inhibits Glioma Single Cell Infiltration and Tumor Growth. *Neuro Oncol* (2013) 15(7):840–52. doi: 10.1093/neuonc/not025
44. Swiatek-Machado K, Mieczkowski J, Ellert-Miklaszewska A, Swierk P, Fokt I, Szymanski S, et al. Novel Small Molecular Inhibitors Disrupt the JAK/STAT3 and FAK Signaling Pathways and Exhibit a Potent Antitumor Activity in Glioma Cells. *Cancer Biol Ther* (2012) 13(8):657–70. doi: 10.4161/cbt.20083
45. Swiatek-Machado K, Kaminska B. STAT Signaling in Glioma Cells. *Adv Exp Med Biol* (2020) 1202:203–22. doi: 10.1007/978-3-030-30651-9_10
46. Guo YJ, Pan WW, Liu SB, Shen ZF, Xu Y, Hu LL. ERK/MAPK Signalling Pathway and Tumorigenesis. *Exp Ther Med* (2020) 19(3):1997–2007. doi: 10.3892/etm.2020.8454
47. Padfield E, Ellis HP, Kurian KM. Current Therapeutic Advances Targeting EGFR and EGFRvIII in Glioblastoma. *Front Oncol* (2015) 5:5. doi: 10.3389/fonc.2015.00005
48. Fu Y, Xin Z, Ling Z, Xie H, Xiao T, Shen X, et al. A CREB1-miR-181a-5p Loop Regulates the Pathophysiologic Features of Bone Marrow Stromal Cells in Fibrous Dysplasia of Bone. *Mol Med* (2021) 27(1):81. doi: 10.1186/s10020-021-00341-z
49. Urabe F, Kosaka N, Ito K, Kimura T, Egawa S, Ochiya T. Extracellular Vesicles as Biomarkers and Therapeutic Targets for Cancer. *Am J Physiol Cell Physiol* (2020) 318(1):C29–39. doi: 10.1152/ajpcell.00280.2019
50. Abels ER, Breakefield XO. Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake. *Cell Mol Neurobiol* (2016) 36(3):301–12. doi: 10.1007/s10571-016-0366-z
51. Nazimek K, Bryniarski K. Perspectives in Manipulating EVs for Therapeutic Applications: Focus on Cancer Treatment. *Int J Mol Sci* (2020) 21(13):4623. doi: 10.3390/ijms21134623

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

7.2 Identification of Dysregulated microRNAs in Glioblastoma Stem-Like Cells

brain sciences

MDPI

Communication

Identification of Dysregulated microRNAs in Glioblastoma Stem-Like Cells

Lara Evers [1,†], Agnes Schäfer [1,†], Raffaella Pini [2], Kai Zhao [1], Susanne Stei [1], Christopher Nimsky [1,3] and Jörg W. Bartsch [1,3,*]

[1] Department of Neurosurgery, Philipps-University Marburg, University Hospital Marburg (UKGM), Baldingerstrasse, 35043 Marburg, Germany
[2] Center for Omics Sciences, IRCCS San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy
[3] Marburg Center for Mind, Brain and Behavior (MCMBB), 35032 Marburg, Germany
* Correspondence: jbartsch@med.uni-marburg.de
† These authors contributed equally to this work.

Abstract: Glioblastoma multiforme (GBM) is the most common malignant primary brain tumor in adults. Despite multimodal therapy, median survival is poor at 12–15 months. At the molecular level, radio-/chemoresistance and resulting tumor progression are attributed to a small fraction of tumor cells, termed glioblastoma stem-like cells (GSCs). These CD133-expressing, self-renewing cells display the properties of multi-lineage differentiation, resulting in the heterogenous composition of GBM. MicroRNAs (miRNAs) as regulators of gene expression at the post-transcriptional level can alter many pathways pivotal to cancer stem cell fate. This study explored changes in the miRNA expression profiles in patient-derived GSCs altered on differentiation into glial fiber acid protein (GFAP)-expressing, astrocytic tumor cells using a polymerase chain reaction (PCR) array. Initially, 22 miRNAs showed higher expression in GSCs and 9 miRNAs in differentiated cells. The two most downregulated miRNAs in differentiated GSCs were miR-17-5p and miR-425-5p, whilst the most upregulated miRNAs were miR-223-3p and let-7-5p. Among those, miR-425-5p showed the highest consistency in an upregulation in all three GSCs. By transfection of a 425-5p miRNA mimic, we demonstrated downregulation of the GFAP protein in differentiated patient-derived GBM cells, providing potential evidence for direct regulation of miRNAs in the GSC/GBM cell transition.

Keywords: glioblastoma multiforme; glioblastoma stem-like cells; differentiation; microRNA; GFAP; miR-425-5p; miR-223-3p; let-7; miR-17-5p

Citation: Evers, L.; Schäfer, A.; Pini, R.; Zhao, K.; Stei, S.; Nimsky, C.; Bartsch, J.W. Identification of Dysregulated microRNAs in Glioblastoma Stem-Like Cells. *Brain Sci.* **2023**, *13*, 350. https://doi.org/10.3390/brainsci13020350

Academic Editors: Agata Grazia D'Amico, Celeste Caruso Bavisotto and Assunta Virtuoso

Received: 12 December 2022
Revised: 13 February 2023
Accepted: 16 February 2023
Published: 18 February 2023

1. Introduction

Glioblastoma multiforme, the most common malignant primary brain tumor in adults, is characterized by an aggressive and invasive growth pattern, rapid development of radio-/chemoresistance, and genetic heterogeneity [1]. The current therapeutic standard of care consists of maximum safe surgical resection, radiation, and temozolomide (TMZ) chemotherapy [2]. However, the median survival remains low at 12–15 months as tumor recurrence occurs rapidly [3].

Glioblastoma stem-like cells (GSCs) are currently viewed as modulators of the tumor microenvironment as well as the origin of radio-/chemoresistance thereby resulting in tumor progression [4]. Due to this small, but pluripotent self-renewing subpopulation of GBM tumor cells that typically reside in perivascular niches apart from the bulk tumor mass, GSCs cannot be sufficiently targeted by surgical resection [5]. As a result of GBM heterogeneity due to different GBM phenotypes, such as the classical, proneural, and mesenchymal types, patient-derived cell-cultured GSC lines might display diverging characteristics [6]. At the molecular level, GSCs express a unique pattern of stemness markers such as the transmembrane glycoproteins CD44 and CD133 or the transcription factor Sex-determining region Y-box2 (SOX2) [4,7]. In particular, CD133, an established

marker for neural progenitor cells and cancer stem-like cells, organizes the cell membrane topology [8]. Contrary to the bulk mass of astrocyte tumor cells, GSCs barely express the intermediate filament GFAP [9]. Therefore, GFAP is utilized as a marker for primary differentiated, astrocytic tumor cells in GBM [10].

Not only does the expression pattern of proteins change on GSC differentiation but recent studies also suggest that GSCs display a unique miRNA expression pattern [11]. MicroRNAs (miRNAs) as small noncoding RNA molecules regulate gene expression at the posttranscriptional level by binding to and thereby targeting their corresponding mRNAs [12]. As miRNAs can mediate many critical pathways to cancer progression such as proliferation, apoptosis, and angiogenesis, they can act as both tumor-suppressors (tumor-suppressor miRNAs) and oncogenes (onco-miRNAs) [13]. Clinically, miRNAs are receiving rising attention as their function as novel diagnostic and prognostic biomarkers, as well as future therapeutic agents, is discussed and given that they can affect multiple target genes involved in pathological processes [11].

For this reason, dysregulated miRNAs are intensely studied in GBM. However, the expression profile as well as the function of specific miRNAs in GSCs have not yet been adequately elucidated.

This study investigated changes in the miRNA expression profile in patient-derived, well-characterized, cultured, sphere-forming GSCs and their differentiated status as adherent GBM cells by utilizing a miRNA PCR array. As a result, a total of 31 dysregulated miRNAs were identified. Through a literature review and target prediction analyses, we closely investigated the most dysregulated miRNAs.

2. Materials and Methods

2.1. Cell Culture

After approval from the local ethics committee (Philipps University Marburg, medical faculty, file number 185/11), patient-derived GSCs as well as primary GBM cell lines were obtained during surgical resection. Each patient gave written informed consent before surgical resection. Isolation, preparation, and molecular characteristics of GSCs and primary GBM cell lines from resected tumor tissues were described previously [14,15]. GSC lines 2017/151, 2017/74, and 2016/240 were cultivated in non-cell-culture-treated Petri dishes. As a medium, DMEM/F12 (DMEM-12-A, Capricorn Scientific, Ebsdorfergrund, Germany), supplemented with 2% B27 (17504044, Thermo Fisher Scientific, Waltham, MA, USA), 1% amphotericin (15290026, Thermo Fisher Scientific, Waltham, MA, USA), 0.5% HEPES (H0887, Sigma-Aldrich, Taufkirchen, Germany), and 0.1% gentamycin (A2712, Biochrom, Berlin, Germany), was utilized. In addition, epidermal growth factor (EGF, 100-15, Peprotech, Hamburg, Germany) and basic fibroblast growth factor (bFGF, 100-18b, Peprotech, Hamburg, Germany) were both supplemented at a final concentration of 0.02 ng/µL. Primary differentiated GBM cell lines GBM100 and GBM42 were cultivated in phenol red-free DMEM (DMEM-HXRXA, Capricorn Scientific, Ebsdorfergrund, Germany) supplemented with 10% fetal calf serum (FCS, S0615, Sigma, Taufkirchen, Germany), 1% penicillin/streptomycin (2321115, Gibco, Carlsbad, CA, USA), 1 mM sodium pyruvate (NPY-B, Capricorn Scientific, Ebsdorfergrund, Germany), 1% L-glutamine (25030-024, Gibco, Carlsbad, CA, USA), and 1% non-essential amino acids (11140050, Gibco, Carlsbad, CA, USA). All cell lines were cultivated in a humidified atmosphere at 37 °C and 5% CO_2.

2.2. GSC Differentiation

To differentiate GSCs, cells of 2016/240, 2017/151, and 2017/74 were seeded in 6-well plates at a density of 750,000 cells in 2 mL. To initiate GSC differentiation, 10% FCS (S0615, Sigma-Aldrich, Taufkirchen, Germany) was supplemented in DMEM/F12. In addition, bFGF and EGF were withdrawn. After seven days of incubation, light-microscopy images were taken. Then, cells were harvested for further analyses.

2.3. miR-425-5p Mimic Transfection

For transient overexpression of miR-425-5p, primary GBM100 and GBM42 cell lines were transfected with 0.01 µM hsa-miR-425-5p miRCURY LNA miRNA (GeneGlobe ID: YM00471725-ADA, catalog no.: 339173, Qiagen, Hilden, Germany). In detail, cells were seeded in a 6-well format at a density of 300,000 cells in 2 mL. After 24 h of incubation and attaching, the transfection was performed with Lipofectamine 2000 Reagent (11668-030, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Meanwhile, 0.01 µM Allstar Negative Control siRNA (1027280, Qiagen, Hilden, Germany) was transfected as a control. The transfection was repeated after 24 h. Cells were harvested 48 h after the second transfection and further analyzed via qPCR and Western blot.

2.4. RNA and miRNA Isolation

Total RNA with an enriched fraction of miRNAs from cellular pellets was isolated using the miRNeasy Tissue/Cells Advanced Mini Kit (217684, Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.5. RNA Reverse Transcription (RT) and Quantitive Real-Time Polymerase Chain Reaction (qPCR)

To quantify gene expression on an mRNA level, total RNA was reverse transcribed using the RNA to cDNA EcoDryTM Premix (639548, TaKaRa, Saint-Germain-en-Laye, France) according to the manufacturer's instructions. Quantitative real-time PCR was performed with a total reaction volume of 20 µL/well, consisting of 10 µL SYBR Green/Rox Master Mix (PPLUS-R-10 ML, Primer Design, Eastleigh, UK), 2 µL *GFAP/CD133* primers (244900, Qiagen, Hilden, Germany), 6 µL nuclease-free water, and 2 µL cDNA. Expression of the ribosomal gene *RPLP0/XS13* (fw: 5′-TGG GCA AGA ACA CCA TGA TG-3′; rev: 5′-AGT TTC TCC AGA GCT GGG TTG T-3′) was used as a housekeeping gene for normalization [16]. PCR experiments were performed on the Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Relative gene expression was calculated utilizing the $2^{-\Delta\Delta Ct}$ method.

2.6. miRNA Reverse Transcription and miRNA PCR Array

First, pooled samples for GSCs and differentiated GBM cells consisting of 8.3 ng total RNA with an enriched miRNA fraction from each of the three cell lines (2017/151, 2016/240, and 2017/74) were generated. As a next step, reverse transcription of the pooled samples was performed utilizing a miScript II RT Kit (218161, Qiagen, Hilden, Germany). Then, following the manufacturer's instructions, a pathway-focused miRNA PCR array (331221 miScript, Qiagen, Hilden, Germany) was conducted utilizing the miScript SYBR Green PCR Kit (218073, Qiagen, Hilden, Germany). The miRNA PCR arrays were performed on the Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Data analysis and scatter plot generation were performed using the corresponding online data analysis tool provided by Qiagen (miScript miRNA PCR Data Analysis, Qiagen, https://dataanalysis.qiagen.com/mirna/arrayanalysis.php?target=upload, accessed on 1 February 2023). Relative gene expression was calculated utilizing the $2^{-\Delta\Delta Ct}$ method. RNU6 was used for internal normalization. Results are presented in heatmaps, which were generated using the GraphPad PRISM 9 software, version 9.1 (Insight Partners, New York, NY, USA).

2.7. miRNA Reverse Transcription and qPCR

For verification of the miRNA PCR array results and further functional experiments on miR-425-5p, isolated RNA samples with an enriched fraction of miRNAs were reverse transcribed utilizing the miRCURY LNA RT Kit (339340, Qiagen, Hilden, Germany), according to the manufacturer's instructions. Quantitative real-time PCR was performed utilizing the miRCURY LNA SYBR® Green PCR Kit (339345, Qiagen, Hilden, Germany), according to the manufacturer's instructions. As miRNA primers, hsa-miR-17-5p miRCURY LNA miRNA PCR Assay (YP02119304, 339306, Qiagen, Hilden, Germany), hsa-miR-425-5p miRCURY

LNA miRNA PCR Assay (YP00204337, 339306, Qiagen, Hilden, Germany), hsa-miR-223-3p 5p miRCURY LNA miRNA PCR Assay (YP00205986, 339306, Qiagen, Hilden, Germany), and hsa-miR-7a-5p miRCURY LNA miRNA PCR Assay (YP00205727, 339306, Qiagen, Hilden, Germany) were used. For normalization, miR-24-5p (YP00203954, 339306, Qiagen, Hilden, Germany) and UniSp6 (YP00203954, 339306, Qiagen, Hilden, Germany) were used. Relative gene expression was calculated utilizing the $2^{-\Delta\Delta Ct}$ method.

2.8. Protein Extraction and Western Blot Analysis

For 30 min, whole cell lysates were incubated in RIPA buffer (50 mM HEPES pH 7.4; 150 mM NaCl; 1% (*v*/*v*) NP-40; 0.5% (*w*/*v*) natriumdeoxycholate; 0.1% (*w*/*v*) SDS; 10 mM phenantrolin; 10 mM EDTA; PierceTM Protease Inhibitor Mini Tablets, EDTA-free, Thermo Fisher Scientific; PierceTM Phosphatase Inhibitor Mini Tablets, Thermo Fisher Scientific). Then, protein samples were prepared in 5× Laemmli buffer (60 mM Tris HCl, pH: 6.8; 2% (*w*/*v*) SDS; 10% (*w*/*v*) glycerol; 5% (*v*/*v*) ß-mercaptoethanol; 0.01% (*w*/*v*) bromophenol blue) and 10× NuPAGETM sample reducing reagent (Thermo Fisher Scientific, Waltham, MA, USA). To separate proteins, samples were denatured at 95 °C for 5 min, then 12.5% SDS polyacrylamide gel was utilized for separation. Separated proteins were transferred onto nitrocellulose membranes (A29591442, GE Healthcare Life Science, Munich, Germany) followed by blocking in 5% (*w*/*v*) milk powder (MP) in TBST (50 mM Tris, pH 7.5; 150 mM NaCl; 0.1% (*w*/*v*) Tween-20), and then incubated for 1 h. The following primary antibodies were utilized: anti-PTEN (1:1000 in 5% MP in TBST, 9559T, Cell Signaling, Leiden, NL, USA), anti-GFAP (1:1000 in 5% bovine serum albumin in TBST, M0761, Dako GmbH, Jena, Germany), anti-SOX2 (1:2000 in 5% MP in TBST, ab97959, Abcam, Berlin, Germany), and anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase, 1:10,000 in 5% MP in TBST, 181602, Abcam, UK). After overnight incubation with primary antibodies at 4 °C, nitrocellulose membranes were washed three times with TBST. Then, membranes were incubated with horseradish peroxidase-conjugated antibodies (ab2116, Abcam, 1:5000) for 1 h. Membranes were washed again with TBST. By the addition of Western Bright Sirius substrate (K-12043-D10, Advansta, San Jose, CA, USA), chemiluminescence was detected using the ChemiDoc MP Imaging System (Bio-rad Laboratories GmbH, Feldkirchen, Germany). Western blot quantification was realized by using the Image J software version 1.53t (NIH, Bethesda, MD, USA).

2.9. Kyoto Encyclopedia of Genes and Genomes (KEGG) Analyses

To functionally characterize the most dysregulated miRNAs in patient-derived GSCs and their differentiated status, KEGG enrichment analysis was performed using the DIANAmiRPath v3.0 web app, an online software suite dedicated to the evaluation of the regulatory role of miRNAs and the identification of controlled pathways [17]. The barplot and the chord diagram were built in the R environment (v. 4.1.3) with ggplot2, and circlize R packages [18,19].

2.10. Statistical Analysis

Results from multiple replicates are presented as the mean ± standard deviation (SD). The miRNA PCR array was conducted once. Paired Student's *t*-tests were applied for statistical comparison between the two groups. Results were considered as not significant (ns) ($p > 0.05$), significant (*) ($p < 0.05$), highly significant (**) ($p < 0.01$), or very highly significant (***) ($p < 0.001$) / (****) ($p < 0.0001$). Statistical analysis was performed utilizing GraphPad PRISM 9, version 9.1 (Insight Partners, New York, NY, USA).

3. Results

3.1. Differentiation of GSCs

GSC lines 2017/151, 2016/240, and 2017/74 were derived from resected tumor tissues of three patients with primary, isocitrate-dehydrogenase (IDH) wildtype GBM. Information regarding molecular-pathological features as well as clinical information is presented in Table 1. In cell culture, GSCs formed typical non-adherent neurospheres (Figure 1a, left).

On differentiation, cells acquired morphological features similar to those of glial cells. For instance, differentiated cells grew in monolayers attached to the bottom of six-well plates and developed long, star-shaped cellular protrusions (Figure 1a, right). As previously demonstrated, a side population analysis was conducted. Here, a population of cells with a higher efflux, hence a lower intracellular concentration of Hoechst dye, was identified. Inhibition of ABC transporters with verapamil and concomitant blockage of efflux confirmed the specificity of the side population as an efflux was no longer detectable [14]. At the molecular level, GSCs expressed high levels of the stem cell marker *CD133*. In contrast, differentiation resulted in a significant decrease in *CD133* expression on the mRNA level in all three GSC lines (Figure 1b, left). On the mRNA level, additional stem cell markers such as CD44, Sox2, and Nestin were tested with similar, but less consistent trends for Sox2 and Nestin, while CD44 was induced in differentiated GSCs (Supplementary Figure S1), similar to the significant increase observed for *GFAP* mRNA expression (Figure 1b, right).

Table 1. Clinical information and histopathological characteristics of patient-derived GSC lines. All three patients suffered from primary, isocitrate dehydrogenase (IDH) wildtype glioblastoma. Here, clinical parameters including age at diagnosis, sex, survival in days, and tumor localization are presented. Furthermore, histopathological data such as methylation status of the O6-methylguanine-DNA-methyltransferase (MGMT), p53 accumulation, and Ki67 labeling index (Ki67-Li) are presented.

GSC Line	Age at Diagnosis (in Years)	Sex	Survival in Days	Localization	MGMT Promotor Methylation Status	Ki67-Li	p53 Accumulation
2016/240	48	female	641	right frontal lobe	methylated	up to 10%	moderately accumulated
2017/151	66	male	126	right temporal lobe and right insula	methylated	up to 20%	accumulated
2017/74	61	male	398	right temporal lobe	not methylated	up to 50%	moderately accumulated

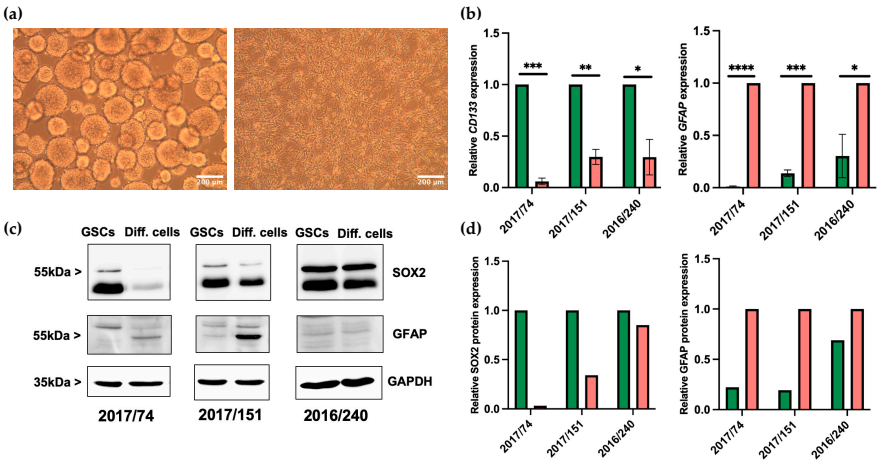

Figure 1. Differentiation of GSCs in astrocytic tumor cells. (**a**) Light microscopy images of 2017/151 spheroid GSCs (left) and adherent differentiated cells (Diff. cells, right). (**b**) Expression of *CD133* and *GFAP* on an mRNA level by RT-qPCR in GSCs (green bars) and corresponding differentiated, astrocytic cells (red bars). Results are given as mean ± SD of three independent experiments. A paired Student's *t*-test was applied to determine significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (**c**) Western Blot of GSCs and Diff. cells showing SOX2 and GFAP expression, where GAPDH was used for internal normalization. (**d**) Western Blot quantification of stem cell marker SOX2 and differentiation marker GFAP in three GSCs lines (green bars) and differentiated cells (red bars).

In addition, Western blot analysis demonstrated that the stem cell marker SOX2 is more greatly expressed in GSCs compared to differentiated cells, while GFAP protein expression increases on differentiation (Figure 1c,d). To summarize, these data suggest that all three GSC lines were successfully differentiated into adherent, growing, astrocytic tumor cells, although to different extents.

3.2. Identification of Dysregulated miRNAs in GSCs and Differentiated Cells

As a screening method to identify changes in miRNA expression induced by GSC differentiation, a pathway-focused miRNA PCR array was conducted. This was realized by generating pooled samples of either GSCs or differentiated cells, containing an equal concentration of miRNAs from each of the three GSC lines and differentiated cell lines, respectively. Differences in miRNA expression based on fold regulation are presented for 84 tested miRNAs by a heatmap (Figure 2a). While green signals represent upregulation in GSCs, the red color indicates the downregulation of respective miRNAs in GSCs and consequently higher expression in differentiated cells. All miRNAs exhibiting fold regulation values > 2 or <−2 were interpreted to be dysregulated by the Qiagen analysis tool. A scatter plot analysis revealed that from a total of 84 tested miRNAs, 22 miRNAs were more greatly expressed in GSCs compared to differentiated cells. In contrast, nine miRNAs displayed lower expression in GSCs and were consequently more greatly expressed in differentiated cells (Figure 2b). A detailed analysis of these dysregulated miRNAs depicted by a heatmap revealed that 10 out of 31 miRNAs were particularly strongly dysregulated (Figure 2c and Table 2). Notably, miR-425-5p, miR-17-5p, miR-424-5p, miR-195-5p, and miR-30c-5p were highly expressed in GSCs. Meanwhile, miR-223-3p and four members of the let-7 miRNA family displayed higher expression in differentiated cells. A thorough literature research was conducted on these 10 miRNAs. Here, we focused on the general role of each miRNA in GBM and the current status of research concerning GSCs (Table 2).

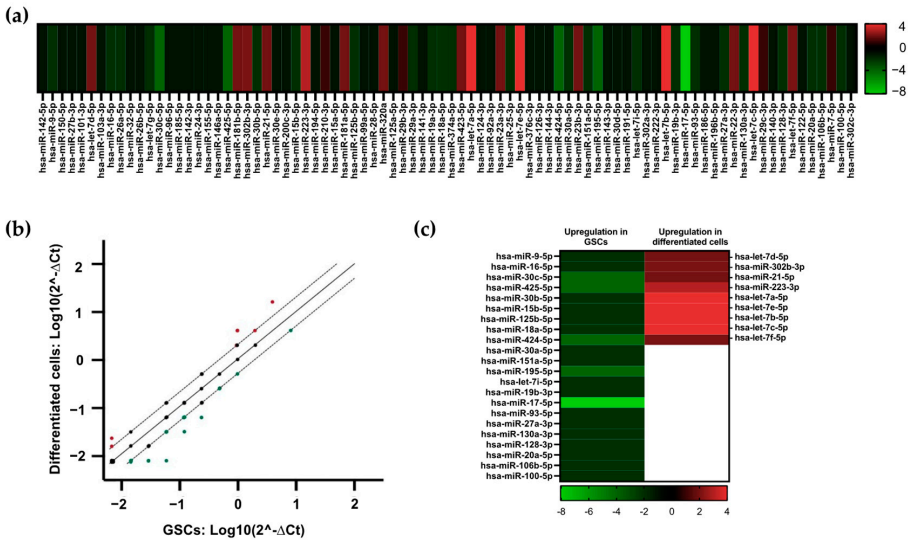

Figure 2. Differentially expressed miRNAs in GSCs and differentiated GBM cells. (**a**) Heatmap of differentially expressed miRNAs in pooled GSCs and pooled differentiated cells generated by fold expression values. Fold regulation values > 1 indicate lower miRNA expression in GSCs and overexpression in differentiated cells (red). Fold regulation values < 0 indicate higher expression in GSCs in comparison with differentiated cells (green). (**b**) Scatter plot analysis (log10 of 2^−delta Ct) of 84 miRNAs tested by the miRNA PCR array. Dotted lines equal log10 of fold regulation of 2 and −2. Green dots indicate upregulation in GSCs, black dots indicate no dysregulation, and red dots indicate overexpression in differentiated cells. (**c**) Heatmap of all tested miRNAs exhibiting fold regulation > 2 or < −2. Fold regulation values > 2 indicate miRNA overexpression in differentiated cells compared to GSCs (red). Fold regulation < −2 indicates miRNA overexpression in GSCs (green).

Table 2. Literature review results on highly dysregulated miRNAs identified in the PCR array. The table includes PCR array results indicated by fold regulation and published data on highly dysregulated miRNAs. We focused on the general role of each miRNA in GBM and their potential roles in GSCs. Plus, reported functionally relevant mRNA targets and predicted target genes directly involved in a GSC or differentiated state, respectively, are listed. For miRNA target prediction, the online software miRPathDB v2.0 was used.

miRNA	Upregulated in	Fold Regulation	Role in GBM	Role in GSCs	mRNA Target
miR-17-5p	GSCs	−8.05	Onco-miRNA [20] Highly expressed in GBM, correlated with poor prognosis [20]	Highly expressed in GSCs [21–23] Increases GSC proliferation [21]	PTEN [23] GFAP (predicted) [24]
miR-425-5p	GSCs	−4.00	Onco-miRNA [25] Associated with poor prognosis [25]	Highly expressed in GSCs [25] Promotes neurosphere formation and GSC survival [25]	PTEN [26] GFAP (predicted) [24]
miR-30c-5p	GSCs	−4.00	Conflicting data Promotes chemoresistance [27] Inhibition of proliferation, migration, and invasion [28] Downregulation in GBM tissue [28]	Unexplored	SOX-9 [28]
miR-424-5p	GSCs	−4.04	Conflicting data Effects on migration and proliferation, induction of apoptosis [29–31] Inhibition of epithelial-to-mesenchymal transition (EMT) and tumor growth [31] Enhances chemoresistance [30]	Unexplored	Akt-1, RAF1 [29] GFAP (predicted) [24]
miR-195-5p	GSCs	−4.05	Conflicting data Affects response to TMZ [32,33] Inhibits proliferation [34] Upregulated in recurrent GBM samples [35]	Unexplored	Cyclin E1 [32] Cyclin D1 [34] GFAP (predicted) [24]
let-7a-5p	Differentiated cells	4.01	Tumor-suppressor miRNA family [36,37] Inhibition of tumor cells' migration, proliferation, and invasion [36,37] Promotes cell cycle arrest and apoptosis [36]	Low expression in GSCs [38] Inhibition of neurosphere growth [37]	K-Ras [36] Musashi-2 (predicted) [24]
let-7e-5p		3.97			MMP9 [39] Musashi-2 (predicted) [24]
let-7b-5p		3.97			E2F2 [37] Musashi-2, Musashi-1 (predicted) [24]
let-7c-5p		4.00			Musashi-2 (predicted) [24]
miR-233-3p	Differentiated cells	3.09	Tumor-suppressor miRNA [40] Enhances radiation sensitivity of GBM cells [40]	Unexplored	ATM [40] Musashi-2 (predicted) [24]

Taken together, the PCR array identified a changed miRNA expression profile in GSCs and differentiated cells and revealed several candidate miRNAs for further investigation into GSC/GBM cell transition.

3.3. miR-425-5p Is Downregulated in Differentiated GSCs

From all dysregulated miRNAs, four miRNAs, miR-425-5p, miR-17-5p, let-7a-5p, and miR-223-3p, turned out to be functional candidates in GSCs for tumor cell differentiation. To validate the miRNA expression levels found in our PCR array screening using pooled miRNAs from all three GSCs (Figure 3a), we determined miRNA expression levels in all three GSC lines separately (Figure 3b and Supplementary Figure S2). Most consistently, miR-425-5p was significantly overexpressed in all three GSC lines compared to differentiated, astrocytic tumor cells (Figure 3b). miR-17-5p proved to be significantly overexpressed in GSCs (Supplementary Figure S2a). Meanwhile, let-7a-5p and miR223-3p downregulation in GSCs compared with differentiated cells could not be verified in the three GSC lines (Supplementary Figure S2b,c).

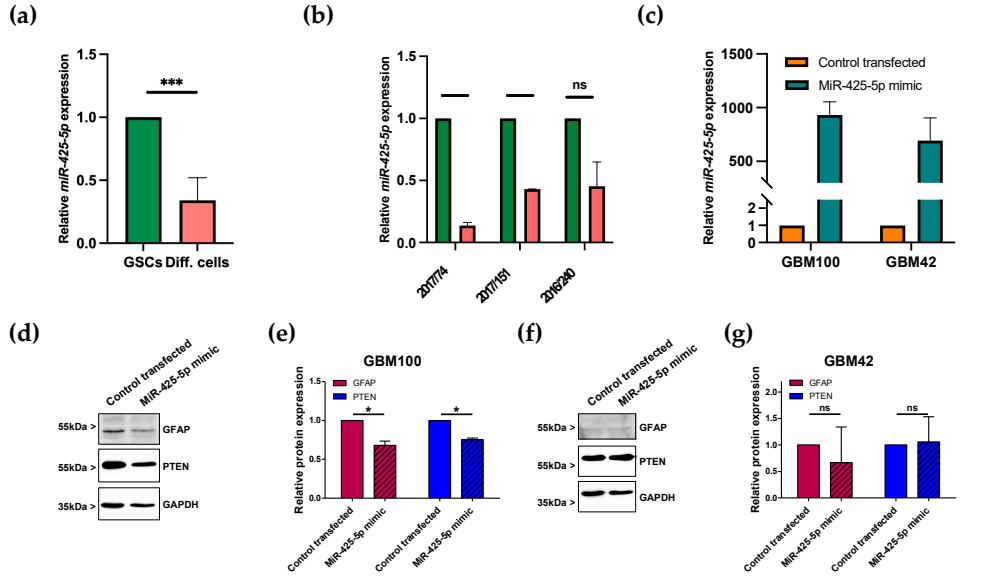

Figure 3. Expression of miR-425-5p in GSCs and effects on GFAP and PTEN protein levels in GBM cells. (**a**) Expression of miR-425-5p in GSCs (green) and their differentiated state (red). RT-qPCR results for differentiated 2017/74, 2017/151, and 2016/240 were normalized to undifferentiated controls. (**b**) Detailed depiction of miR-425-5p expression in each GSC line (green) and its corresponding differentiated state (red). Results are shown as mean values ± SD of two independent experiments. (**c**) miR-425-5p mimic transfection of primary GBM cell lines GBM100 and GBM42. (**d**) Representative Western blot demonstrating GFAP and PTEN expression after miR-425-5p mimic transfection in GBM100 cells. (**e**) Quantification of GFAP and PTEN protein expression in transfected GBM100 cells. (**f**) Representative Western blot demonstrating GFAP and PTEN expression after miR-425-5p mimic transfection in GBM42 cells. (**g**) Quantification of GFAP and PTEN protein expression in transfected GBM42 cells. Two independent experiments were conducted. Results are presented as mean values ± SD. A paired Student's *t*-test was applied to determine significance: ns $p > 0.05$, * $p < 0.05$, *** $p < 0.001$.

3.4. Transfection of miRNA Mimic Affects Protein Levels of GFAP in Patient-Derived GBM Cells

As shown in Table 2, miR-425-5p was identified to potentially target the GFAP gene, which is expressed as a marker of differentiated GBM cells, so that downregulation of miR-425-5p in GSCs could increase GFAP levels in the course of differentiation into GBM cells. To explore this, we transfected two patient-derived GBM cell strains (GBM100 and GBM42) with a miR-425-5p mimic miRNA (Figure 3c). About 48 h after transfection, cells

were lysed and analyzed for protein levels of GFAP and the known miR-425-5p target PTEN. In GBM100, both GFAP and PTEN protein levels were reduced after mimic transfection (Figure 3d,e), whereas the results for GBM42 were inconsistent (Figure 3f,g). Those first results suggested that GFAP is a target gene for miR-425-5p and could potentially regulate the differentiation state of GBM cells.

3.5. miRNA Profiling in GSC Maintenance and Differentiation

The 10 most regulated miRNAs were analyzed for their biological functions by KEGG enrichment analysis (Figure 4a). The most significant pathways were "Signaling pathways regulating pluripotency of stem cells", "Pathways in cancer", and "PI3K-Akt signaling", a pathway of high importance in GBM. In Figure 4b, the miRNA target relationship to pathways regulating the pluripotency of stem cells is shown in the form of a chord plot. All 10 miRNAs are connected to their mRNA targets, thereby showing their contribution to GSC maintenance or differentiation.

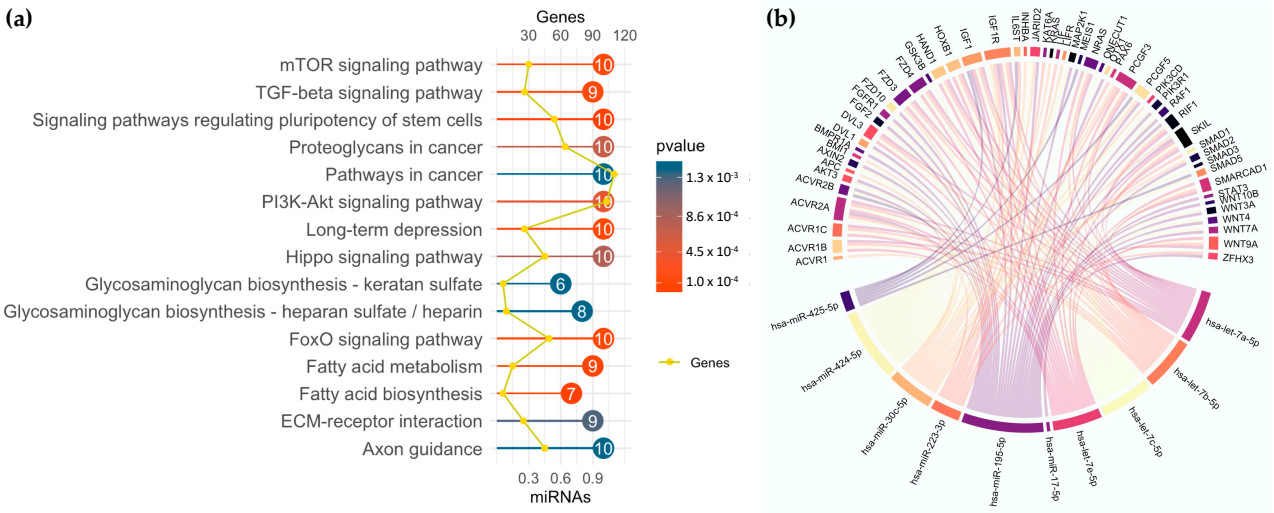

Figure 4. Bioinformatic analysis of the 10 most dysregulated miRNAs. (**a**) KEGG enrichment analysis of the 10 most dysregulated miRNAs. The x-axis reports the number of target genes and the fraction of miRNAs in the starting list involved in the pathway. The number within the dots represents the number of miRNAs. (**b**) The miRNA–target relationship in the KEGG_hsa04550 signaling pathway, "Signaling pathways regulating pluripotency of stem cells". Each link represents a miRNA–target interaction.

4. Discussion

Due to tumor heterogeneity and limited therapeutic options, GBM remains an incurable disease with a devasting prognosis. As modulators of the tumor microenvironment and radio-/chemoresistance, GSCs are considered putative future therapeutic targets [6]. Even though GSCs play a key role in tumor cell invasion, recurrence, and angiogenesis, many underlying signaling pathways remain elusive [41]. MicroRNAs (miRNAs) can act as central regulatory molecules of GBM hallmarks such as invasion or immune evasion [42]. Therefore, miRNAs are discussed as future therapeutic targets and diagnostic biomarkers [43]. Our study detected differences in the miRNA expression profile of GSCs and differentiated tumor cells by PCR array screening. Cultured patient-derived GSCs were stimulated to differentiate into astrocytic tumor cells. Although this in vitro differentiation model is commonly used in GSC research, in vivo GSC differentiation is a complex process as the tumor microenvironment is shaped by a variety of different cell types such as macrophages, microglia, and mesenchymal cells [44].

Based on our results from miRNA PCR array screening, a literature review of mRNA targets, and validating experiments in each GSC line, we present four suitable miRNA

candidates, miR425-5p, miR-17-5p, miR-223-3p, and let-7a-5p, which might be directly and indirectly involved in the regulation of GBM cell differentiation.

Firstly, miR-17-5p, the most dysregulated miRNA in the conducted array, is a known onco-miRNA in GBM [20]. Consistent with other studies, miR-17-5p was highly expressed in GSCs as it stimulates GSC proliferation [21–23]. As physically adjacent miRNA genes are often transcribed at the same time, they are summarized as a cluster. miR-17-5p is often analyzed as a part of the miR-17-92 cluster [45]. Notably, four of six miRNAs of the miR-17-92 cluster were consistently upregulated in our conducted PCR array. In GBM, the miR-17-92 cluster is highly expressed and correlated with a poor prognosis [20].

Secondly, miR-425-5p was upregulated in GSCs concordant with a study by La Rocha et al. [25]. MiR-425-5p is overexpressed in GBM tissue specimens in comparison to normal brain control specimens and acts as an onco-miRNA [25]. Both miR17-5p and miR-425-5p are known to target phosphatase and tensin homolog (PTEN) mRNA [23,26]. PTEN, a key tumor suppressor, is commonly mutated in GBM carcinogenesis [46]. As a predicted mRNA target based on miRPathDB v2.0, expression levels of GFAP could be regulated directly by miR-425-5p and miR-17-5p, suggesting that these miRNAs control astrocytic cell differentiation [24]. As an experimental proof, mimic miR-425-5p when transfected into differentiated patient-derived GBM cells, can regulate GFAP expression. Since these cells are also able to de-differentiate into GSCs, we interpret our findings to reveal that miR-425-5p is able to contribute, among other critical proteins, to GBM cell differentiation. Moreover, as chord analysis suggests, miR-425-5p is potentially involved in gene regulation of IGF1 (insulin growth factor 1), gp 130 (IL6ST), MEIS1 (a homeobox gene), SMAD5 (TGF-ß pathway), and PCGF5 (a polycomb transcription factor). All of them could be important mediators of growth signals and cell fate determination in GBM cells.

Furthermore, PCR array analysis identified nine miRNAs that were upregulated in differentiated cells. Notably, six of these nine miRNAs are members of the let-7 miRNA family. Published data revealed that the let-7 family acts as a tumor suppressor in GBM [11,36]. Overexpression of let-7 miRNAs leads to the inhibition of tumor cell migration and promotes apoptosis [36]. According to our PCR array, Degrauwe et al. demonstrated that the let-7 family is scarcely expressed in GSCs [38]. As an interesting mRNA target, Kirsten rat sarcoma virus oncogene homolog (K-Ras), an oncogene and activator of its downstream targets in the mitogen-activated protein kinase (MAPK) pathway, was identified [38]. According to miRPathDB v.2.0 target gene prediction, the stem cell marker Musashi-2 can be directly regulated by the let-7 family and miR-223-3p so that high expression of these miRNAs could suppress the GSC phenotype [24,47]. Additionally, miR-223-3p was overexpressed in differentiated cells. In GBM, miR-223-3p functions as a tumor-suppressor miRNA; its overexpression enhances radio-/chemosensitivity in cell culture models as miR-223-3p targets ataxia telangiectasia mutated (ATM) [40]. ATM initiates repair mechanisms after radio-/chemotherapy-induced DNA damage and thereby contributes to radio-/chemoresistance [48]. Since the role of miR-223-3p is currently uninvestigated in GSCs, future detailed analysis of miR-223-3p and ATM in GSCs is justified.

5. Conclusions

Our study detected a changed miRNA expression profile on GSC differentiation in a well-defined in vitro setup. Through a miRNA PCR array, 31 dysregulated miRNAs were identified. About 10 highly regulated miRNAs, including miR-425-5p, miR-17-5p, miR-223-3p, and the let-7 miRNA family, are promising miRNA candidates for further investigations aiming to manipulate the differentiation status of GSCs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/brainsci13020350/s1, Figure S1: qPCR analysis of stem cell markers CD44, Sox2, and Nestin; Figure S2: qPCR analysis of miR425-5p, miR17-5p, let-7a-5p, and miR223-3p in individual GSC lines.

Author Contributions: Conceptualization, J.W.B.; methodology, L.E., A.S. and R.P; investigation, L.E., A.S., K.Z. and S.S.; resources, J.W.B. and C.N.; data curation, L.E., A.S. and R.P.; visualization, L.E., A.S. and R.P.; writing—original draft preparation, L.E.; writing—review and editing, J.W.B. and A.S.; supervision, J.W.B.; project administration, L.E.; funding acquisition, J.W.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the framework of ERANET PerMed joint call 2018, project "PerProGlio", supported by the Federal Ministry for Education and Research (BMBF), grant number 01KU1915B. K.Z. was funded by a CSC scholarship. Open-access funding was provided by the Open Access Publication Fund of Philipps-Universität Marburg with the support of the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation).

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Philipps-Universität Marburg medical faculty (file number 185/11, 12 February 2012) for studies involving tumor material for the preparation of glioblastoma stem-like cells.

Informed Consent Statement: Informed, written consent was obtained from all subjects involved in the study prior to surgical tumor resection.

Data Availability Statement: Data presented in this study are available on reasonable request.

Acknowledgments: The authors wish to thank UKGM for support, and Axel Pagenstecher for expert help.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Vollmann-Zwerenz, A.; Leidgens, V.; Feliciello, G.; Klein, C.A.; Hau, P. Tumor Cell Invasion in Glioblastoma. *Int. J. Mol. Sci.* **2020**, *21*, 1932. [CrossRef] [PubMed]
2. Anjum, K.; Shagufta, B.I.; Abbas, S.Q.; Patel, S.; Khan, I.; Shah, S.A.A.; Akhter, N.; Hassan, S.S.U. Current status and future therapeutic perspectives of glioblastoma multiforme (GBM) therapy: A review. *Biomed. Pharmacother.* **2017**, *92*, 681–689. [CrossRef] [PubMed]
3. Alifieris, C.; Trafalis, D.T. Glioblastoma multiforme: Pathogenesis and treatment. *Pharmacol. Ther.* **2015**, *152*, 63–82. [CrossRef] [PubMed]
4. Garnier, D.; Renoult, O.; Alves-Guerra, M.-C.; Paris, F.; Pecqueur, C. Glioblastoma Stem-Like Cells, Metabolic Strategy to Kill a Challenging Target. *Front. Oncol.* **2019**, *9*, 118. [CrossRef]
5. Lathia, J.D.; Mack, S.C.; Mulkearns-Hubert, E.E.; Valentim, C.L.L.; Rich, J.N. Cancer stem cells in glioblastoma. *Genes Dev.* **2015**, *29*, 1203–1217. [CrossRef]
6. Gimple, R.C.; Bhargava, S.; Dixit, D.; Rich, J.N. Glioblastoma stem cells: Lessons from the tumor hierarchy in a lethal cancer. *Genes Dev.* **2019**, *33*, 591–609. [CrossRef] [PubMed]
7. Aghajani, M.; Mansoori, B.; Mohammadi, A.; Asadzadeh, Z.; Baradaran, B. New emerging roles of CD133 in cancer stem cell: Signaling pathway and miRNA regulation. *J. Cell. Physiol.* **2019**, *234*, 21642–21661. [CrossRef]
8. Barzegar Behrooz, A.; Syahir, A.; Ahmad, S. CD133: Beyond a cancer stem cell biomarker. *J. Drug Target.* **2019**, *27*, 257–269. [CrossRef]
9. Ricci-Vitiani, L.; Pallini, R.; Larocca, L.M.; Lombardi, D.G.; Signore, M.; Pierconti, F.; Petrucci, G.; Montano, N.; Maira, G.; de Maria, R. Mesenchymal differentiation of glioblastoma stem cells. *Cell Death Differ.* **2008**, *15*, 1491–1498. [CrossRef]
10. Aldaz, B.; Sagardoy, A.; Nogueira, L.; Guruceaga, E.; Grande, L.; Huse, J.T.; Aznar, M.A.; Díez-Valle, R.; Tejada-Solís, S.; Alonso, M.M.; et al. Involvement of miRNAs in the differentiation of human glioblastoma multiforme stem-like cells. *PLoS ONE* **2013**, *8*, e77098. [CrossRef]
11. Shea, A.; Harish, V.; Afzal, Z.; Chijioke, J.; Kedir, H.; Dusmatova, S.; Roy, A.; Ramalinga, M.; Harris, B.; Blancato, J.; et al. MicroRNAs in glioblastoma multiforme pathogenesis and therapeutics. *Cancer Med.* **2016**, *5*, 1917–1946. [CrossRef]
12. O'Brien, J.; Hayder, H.; Zayed, Y.; Peng, C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front. Endocrinol.* **2018**, *9*, 402. [CrossRef] [PubMed]
13. Buruiană, A.; Florian, Ș.I.; Florian, A.I.; Timiș, T.-L.; Mihu, C.M.; Miclăuș, M.; Oșan, S.; Hrapșa, I.; Cataniciu, R.C.; Farcaș, M.; et al. The Roles of miRNA in Glioblastoma Tumor Cell Communication: Diplomatic and Aggressive Negotiations. *Int. J. Mol. Sci.* **2020**, *21*, 1950. [CrossRef] [PubMed]
14. Hannen, R.; Selmansberger, M.; Hauswald, M.; Pagenstecher, A.; Nist, A.; Stiewe, T.; Acker, T.; Carl, B.; Nimsky, C.; Bartsch, J.W. Comparative Transcriptomic Analysis of Temozolomide Resistant Primary GBM Stem-Like Cells and Recurrent GBM Identifies Up-Regulation of the Carbonic Anhydrase CA2 Gene as Resistance Factor. *Cancers* **2019**, *11*, 921. [CrossRef]

15. Dong, F.; Eibach, M.; Bartsch, J.W.; Dolga, A.M.; Schlomann, U.; Conrad, C.; Schieber, S.; Schilling, O.; Biniossek, M.L.; Culmsee, C.; et al. The metalloprotease-disintegrin ADAM8 contributes to temozolomide chemoresistance and enhanced invasiveness of human glioblastoma cells. *Neuro Oncol.* **2015**, *17*, 1474–1485. [CrossRef] [PubMed]
16. Schlomann, U.; Koller, G.; Conrad, C.; Ferdous, T.; Golfi, P.; Garcia, A.M.; Höfling, S.; Parsons, M.; Costa, P.; Soper, R.; et al. ADAM8 as a drug target in pancreatic cancer. *Nat. Commun.* **2015**, *6*, 6175. [CrossRef]
17. Vlachos, I.S.; Zagganas, K.; Paraskevopoulou, M.D.; Georgakilas, G.; Karagkouni, D.; Vergoulis, T.; Dalamagas, T.; Hatzigeorgiou, A.G. DIANA-miRPath v3.0: Deciphering microRNA function with experimental support. *Nucleic Acids Res.* **2015**, *43*, W460–W466. [CrossRef]
18. Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*; Springer International Publishing: New York, NY, USA, 2016; ISBN 978-3-319-24275-0.
19. Gu, Z.; Gu, L.; Eils, R.; Schlesner, M.; Brors, B. circlize Implements and enhances circular visualization in R. *Bioinformatics* **2014**, *30*, 2811–2812. [CrossRef] [PubMed]
20. Gruszka, R.; Zakrzewska, M. The Oncogenic Relevance of miR-17-92 Cluster and Its Paralogous miR-106b-25 and miR-106a-363 Clusters in Brain Tumors. *Int. J. Mol. Sci.* **2018**, *19*, 879. [CrossRef]
21. Ernst, A.; Campos, B.; Meier, J.; Devens, F.; Liesenberg, F.; Wolter, M.; Reifenberger, G.; Herold-Mende, C.; Lichter, P.; Radlwimmer, B. De-repression of CTGF via the miR-17-92 cluster upon differentiation of human glioblastoma spheroid cultures. *Oncogene* **2010**, *29*, 3411–3422. [CrossRef]
22. Schraivogel, D.; Weinmann, L.; Beier, D.; Tabatabai, G.; Eichner, A.; Zhu, J.Y.; Anton, M.; Sixt, M.; Weller, M.; Beier, C.P.; et al. CAMTA1 is a novel tumour suppressor regulated by miR-9/9* in glioblastoma stem cells. *EMBO J.* **2011**, *30*, 4309–4322. [CrossRef]
23. Li, H.; Yang, B.B. Stress response of glioblastoma cells mediated by miR-17-5p targeting PTEN and the passenger strand miR-17-3p targeting MDM2. *Oncotarget* **2012**, *3*, 1653–1668. [CrossRef]
24. Kehl, T.; Kern, F.; Backes, C.; Fehlmann, T.; Stöckel, D.; Meese, E.; Lenhof, H.-P.; Keller, A. miRPathDB 2.0: A novel release of the miRNA Pathway Dictionary Database. *Nucleic Acids Res.* **2020**, *48*, D142–D147. [CrossRef]
25. De La Rocha, A.M.A.; González-Huarriz, M.; Guruceaga, E.; Mihelson, N.; Tejada-Solís, S.; Díez-Valle, R.; Martínez-Vélez, N.; Fueyo, J.; Gomez-Manzano, C.; Alonso, M.M.; et al. miR-425-5p, a SOX2 target, regulates the expression of FOXJ3 and RAB31 and promotes the survival of GSCs. *Arch. Clin. Biomed. Res.* **2020**, *4*, 221–238. [CrossRef]
26. Zhou, J.-S.; Yang, Z.-S.; Cheng, S.-Y.; Yu, J.-H.; Huang, C.-J.; Feng, Q. miRNA-425-5p enhances lung cancer growth via the PTEN/PI3K/AKT signaling axis. *BMC Pulm. Med.* **2020**, *20*, 223. [CrossRef] [PubMed]
27. Quintavalle, C.; Donnarumma, E.; Iaboni, M.; Roscigno, G.; Garofalo, M.; Romano, G.; Fiore, D.; de Marinis, P.; Croce, C.M.; Condorelli, G. Effect of miR-21 and miR-30b/c on TRAIL-induced apoptosis in glioma cells. *Oncogene* **2013**, *32*, 4001–4008. [CrossRef] [PubMed]
28. Liu, S.; Li, X.; Zhuang, S. miR-30c Impedes Glioblastoma Cell Proliferation and Migration by Targeting SOX9. *Oncol. Res.* **2019**, *27*, 165–171. [CrossRef]
29. Gheidari, F.; Arefian, E.; Adegani, F.J.; Kalhori, M.R.; Seyedjafari, E.; Kabiri, M.; Teimoori-Toolabi, L.; Soleimani, M. miR-424 induces apoptosis in glioblastoma cells and targets AKT1 and RAF1 oncogenes from the ERBB signaling pathway. *Eur. J. Pharmacol.* **2021**, *906*, 174273. [CrossRef] [PubMed]
30. Xu, H.; Zhang, H.; Tan, L.; Yang, Y.; Wang, H.; Zhao, Q.; Lu, J. FAM87A as a Competing Endogenous RNA of miR-424-5p Suppresses Glioma Progression by Regulating PPM1H. *Comput. Math. Methods Med.* **2021**, *2021*, 7952922. [CrossRef] [PubMed]
31. Zhao, C.; Wang, X.-B.; Zhang, Y.-H.; Zhou, Y.-M.; Yin, Q.; Yao, W.-C. MicroRNA-424 inhibits cell migration, invasion and epithelial-mesenchymal transition in human glioma by targeting KIF23 and functions as a novel prognostic predictor. *Eur. Rev. Med. Pharmacol. Sci.* **2018**, *22*, 6369–6378. [CrossRef]
32. Wang, H.; Ren, S.; Xu, Y.; Miao, W.; Huang, X.; Qu, Z.; Li, J.; Liu, X.; Kong, P. MicroRNA-195 reverses the resistance to temozolomide through targeting cyclin E1 in glioma cells. *Anticancer. Drugs* **2019**, *30*, 81–88. [CrossRef]
33. Xu, N.; Liu, B.; Lian, C.; Doycheva, D.M.; Fu, Z.; Liu, Y.; Zhou, J.; He, Z.; Yang, Z.; Huang, Q.; et al. Long noncoding RNA AC003092.1 promotes temozolomide chemosensitivity through miR-195/TFPI-2 signaling modulation in glioblastoma. *Cell Death Dis.* **2018**, *9*, 1139. [CrossRef]
34. Liu, K.; Deng, Y.; Yang, Y.; Wang, H.; Zhou, P. MicorRNA-195 links long non-coding RNA SEMA3B antisense RNA 1 (head to head) and cyclin D1 to regulate the proliferation of glioblastoma cells. *Bioengineered* **2022**, *13*, 8798–8805. [CrossRef] [PubMed]
35. Yu, W.; Liang, X.; Li, X.; Zhang, Y.; Sun, Z.; Liu, Y.; Wang, J. MicroRNA-195: A review of its role in cancers. *OncoTargets Ther.* **2018**, *11*, 7109–7123. [CrossRef]
36. Wang, X.-R.; Luo, H.; Li, H.-L.; Cao, L.; Wang, X.-F.; Yan, W.; Wang, Y.-Y.; Zhang, J.-X.; Jiang, T.; Kang, C.-S.; et al. Overexpressed let-7a inhibits glioma cell malignancy by directly targeting K-ras, independently of PTEN. *Neuro Oncol.* **2013**, *15*, 1491–1501. [CrossRef]
37. Song, H.; Zhang, Y.; Liu, N.; Zhang, D.; Wan, C.; Zhao, S.; Kong, Y.; Yuan, L. Let-7b inhibits the malignant behavior of glioma cells and glioma stem-like cells via downregulation of E2F2. *J. Physiol. Biochem.* **2016**, *72*, 733–744. [CrossRef]
38. Degrauwe, N.; Schlumpf, T.B.; Janiszewska, M.; Martin, P.; Cauderay, A.; Provero, P.; Riggi, N.; Suvà, M.-L.; Paro, R.; Stamenkovic, I. The RNA Binding Protein IMP2 Preserves Glioblastoma Stem Cells by Preventing let-7 Target Gene Silencing. *Cell Rep.* **2016**, *15*, 1634–1647. [CrossRef] [PubMed]

39. Ventayol, M.; Viñas, J.L.; Sola, A.; Jung, M.; Brüne, B.; Pi, F.; Mastora, C.; Hotter, G. miRNA let-7e targeting MMP9 is involved in adipose-derived stem cell differentiation toward epithelia. *Cell Death Dis.* **2014**, *5*, e1048. [CrossRef] [PubMed]
40. Liang, L.; Zhu, J.; Zaorsky, N.G.; Deng, Y.; Wu, X.; Liu, Y.; Liu, F.; Cai, G.; Gu, W.; Shen, L.; et al. MicroRNA-223 enhances radiation sensitivity of U87MG cells in vitro and in vivo by targeting ataxia telangiectasia mutated. *Int. J. Radiat. Oncol. Biol. Phys.* **2014**, *88*, 955–960. [CrossRef]
41. Boyd, N.H.; Tran, A.N.; Bernstock, J.D.; Etminan, T.; Jones, A.B.; Gillespie, G.Y.; Friedman, G.K.; Hjelmeland, A.B. Glioma stem cells and their roles within the hypoxic tumor microenvironment. *Theranostics* **2021**, *11*, 665–683. [CrossRef]
42. Karsy, M.; Arslan, E.; Moy, F. Current Progress on Understanding MicroRNAs in Glioblastoma Multiforme. *Genes Cancer* **2012**, *3*, 3–15. [CrossRef] [PubMed]
43. He, B.; Zhao, Z.; Cai, Q.; Zhang, Y.; Zhang, P.; Shi, S.; Xie, H.; Peng, X.; Yin, W.; Tao, Y.; et al. miRNA-based biomarkers, therapies, and resistance in Cancer. *Int. J. Biol. Sci.* **2020**, *16*, 2628–2647. [CrossRef]
44. Da Ros, M.; de Gregorio, V.; Iorio, A.; Giunti, L.; Guidi, M.; de Martino, M.; Genitori, L.; Sardi, I. Glioblastoma Chemoresistance: The Double Play by Microenvironment and Blood-Brain Barrier. *Int. J. Mol. Sci.* **2018**, *19*, 2879. [CrossRef]
45. Olive, V.; Jiang, I.; He, L. mir-17-92, a cluster of miRNAs in the midst of the cancer network. *Int. J. Biochem. Cell Biol.* **2010**, *42*, 1348–1354. [CrossRef] [PubMed]
46. Montano, N. Biomarkers in Glioblastoma multiforme. Evidences from a literature review. *J. Clin. Transl. Res.* **2016**, *2*, 3–10. [CrossRef]
47. Kudinov, A.E.; Karanicolas, J.; Golemis, E.A.; Boumber, Y. Musashi RNA-Binding Proteins as Cancer Drivers and Novel Therapeutic Targets. *Clin. Cancer Res.* **2017**, *23*, 2143–2153. [CrossRef]
48. Ferri, A.; Stagni, V.; Barilà, D. Targeting the DNA Damage Response to Overcome Cancer Drug Resistance in Glioblastoma. *Int. J. Mol. Sci.* **2020**, *21*, 4910. [CrossRef]

8. Appendix

8.1 Lebenslauf

8.2 Verzeichnis der akademischen Lehrer/-innen

Meine akademischen Lehrenden waren die Damen und Herren in Marburg: Adeberg, Aigner, Albers, Alter, Baranovski, Bartsch, Bauer, Becker, Becker, Bein, Bertoune, Besgen, Bogdan, Bösner, Burchert, Carl, Cetin, Czubayko, Decher, Denkert, Dietz, Donner-Banzhoff, Falkenberg, Feuser, Frink, Geisel, Geks, Geraedts, Gesche, Görg, Grgic, Gress, Hertl, Hoch, Holzer, Hoyer, Josephs, Kann, Kemmling, Kircher, Kirschbaum, Kluge, Koehler, Kruse, Kühnert, Lill, Lohoff, Lüsebrink, Luster, Mahnken, Mann, Markus, Michel, Milani, Mirow, Moll, Neff, Nenadic, Neubauer, Nimsky, Obermayr, Oberwinkler, Oliver, Opitz, Pagenstecher, Pedrosa, Peterlein, Plant, Pöttgen, Preisig-Müller, Prinz, Rastan, Reese, Renz, Riera-Knorrenschild, Rinke, Rost, Ruchholtz, Rust, Saß, Schäfer, Schieffer, Schmeck, Schu, Schulze, Schulze, Schumacher, Schwabe, Seitz, Sekundo, Sevinc, Simon, Sommer, Stahl, Stuck, Thieme, Timmermann, Torossian, Visser, Vogelmeier, Vogt, Völlger, vom Hofe, Vorwerk, Wagner, Weber, Weihe, Weise, Westermann, Wrocklage, Wulf.

8.3 Danksagung

An dieser Stelle möchte ich mich bei allen Menschen bedanken, die mir ermöglicht haben, diese Arbeit anzufertigen und mich während der letzten Jahre unterstützt haben.

An erster Stelle möchte ich mich bei meinem Doktorvater Prof. Dr. Jörg-Walter Bartsch für die großartige Betreuung in den letzten Jahren bedanken. Du hast mich zuverlässig durch alle Phasen dieser Arbeit begleitet und ich bin dir zutiefst dankbar für dein Vertrauen in mich und deine konstante Unterstützung bei den unzähligen wissenschaftlichen Fragestellungen und Problemen, die während der Erarbeitung dieser These aufgetreten sind.

Zudem möchte ich mich bei Herrn Prof. Dr. Nimsky für den wissenschaftlichen Austausch und die Unterstützung, insbesondere während des Entstehungsprozesses der zweiten Publikation, bedanken.

Ich möchte mich ganz herzlich bei allen aus dem molekularbiologischen Labor der Neurochirurgie für die viele Hilfe und den unkomplizierten Austausch bedanken. Insbesondere geht mein herzlicher Dank an Lena Cook, Susanne Stei, Khai Zhao, Zhuo Zhan und Gian-Luca Dreizner, ihr habt mich unzählige Male bei der Durchführung von Experimenten und dem Lösen von Problemen tatkräftig unterstützt. Ich konnte während meiner Zeit im Labor viele neue Methoden ausprobieren und denke gerne an gemeinsame Mittagessen und Ausflüge zurück.
Liebe Agnes, auch bei dir möchte ich mich für die außerordentliche Unterstützung während meiner Zeit im Labor, und auch danach, bedanken. Du hast immer ein offenes Ohr für all meine wissenschaftlichen Fragen aber auch weit darüber hinaus. Ich konnte sehr viel von dir im Laboralltag lernen und freue mich sehr, dass wir gemeinsam diese beiden Publikationen verfassen konnten.

Abschließend danke ich meiner Familie für die bedingungslose Unterstützung und Liebe in all den Jahren. Ihr habt mich immer ermuntert meine Träume zu verfolgen, ohne euch wäre ich nicht an diesem Punkt gekommen. Außerdem möchte ich mich bei meinen Freunden und Freundinnen für den konstanten Rückhalt und die vielen tollen Momente während unserer Studienzeit bedanken. Ihr habt euch immer Zeit genommen mir zuzuhören und habt mir den Rücken in den schwierigeren Phasen dieser Doktorarbeit gestärkt.

8.4 Ehrenwörtliche Erklärung