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ADAM8 affects glioblastoma progression through microglia/macrophage by regulating osteopontin-mediated angiogenesis

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This cumulative dissertation represents a summary of the research results published in the following two peer-reviewed articles:

[1] Gjorgjevski M, Hannen R, Carl B, **Li Y**, Landmann E, Buchholz M, Bartsch JW, Nimsky C. Molecular profiling of the tumor microenvironment in glioblastoma patients: correlation of microglia/macrophage polarization state with metalloprotease expression profiles and survival. Biosci Rep. 2019 Jun 20;39(6):BSR20182361. doi: 10.1042/BSR20182361. PMID: 31142630; PMCID: PMC6616040.

[2] **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. ADAM8 affects glioblastoma progression by regulating osteopontin-mediated angiogenesis. Biol Chem. 2020 Sep 11:/j/bchm.ahead-of-print/hsz-2020-0184/hsz-2020-0184.xml. doi: 10.1515/hsz-2020-0184. Epub ahead of print. PMID: 32845856.

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1. List of abbreviations

ADAM	A disintegrin and metalloproteases	HUVEC	Human Umbilical Vein endothelial cells
ARG1	Arginase 1	MRI	Magnetic resonance imaging
BBB	Blood-brain barrier	OPN	Osteopontin
BSP-1	Bone sialoprotein 1	pSTAT3	Phosphorylated STAT3
CT	Computed tomography	qPCR	Quantitative PCR
GAMMs	Glioma-associated macrophages/microglia	shRNA	Small hairpin loop RNAs
GBM	Glioblastoma	SPP1	Secreted phosphoprotein 1
HGG	High-grade glioma	TMZ	Temozolomide

2. Table of figures

3. Abstract – Zusammenfassung

3.1 Abstract

This cumulative dissertation summarizes two peer-reviewed publications addressing the role of a Disintegrin and Metalloproteases 8 (ADAM8) in Glioblastoma multiforme (GBM) cells and in the GBM tumor microenvironment.

The first publication entitled “Molecular profiling of the tumor microenvironment in glioblastoma patients: correlation of microglia/macrophage polarization state with metalloprotease expression profiles and survival”. In this study, we investigated the molecular profile of glioma-associated macrophages/microglia (GAMMs) in correlation with patient prognosis by exploiting M1/M2-like polarization markers in a cohort of 20 GBM patients. Using quantitative PCR (qPCR), the markers CXCL10 (M1) and CCL13 (M2) were validated in human macrophages and applied to a global analysis of GBM tissue. Furthermore, proteinase genes, known to be associated with GBM progression (*ADAM8*, *MMP9*, *MMP14*, *ADAM10*, *ADAM17*), were analyzed in correlation to M1/M2 markers. Our data showed that the M2-like macrophage marker CCL13 showed significantly higher expression levels than all M1-like macrophage markers. Genes for *MMP9* and *MMP14* are significantly associated with an M2-like phenotype and association to impaired prognosis in the GBM patient cohort. A high expression in MMP9 and MMP14 is significantly correlated ($P<0.001$) with high expression of ADAM8 in GBM.

The second publication entitled “ADAM8 affects glioblastoma progression by regulating osteopontin-mediated angiogenesis” investigated the functional impact of ADAM8 on GBM progression *in vivo* by stereotactic injection of *ADAM8*-deficient glioma cells into the brains of nude mice (*nu/nu*). Our results provide evidence for a significant contribution of ADAM8 during tumor angiogenesis, as demonstrated *in*

vivo and *in vitro* by functional analysis of *ADAM8*-deficient U87 glioma cells and by analyzing primary macrophages isolated from *Adam8* knockout mice. Notably, mice lacking ADAM8 have no evident developmental or pathological defects as confirmed by phenotyping report of ADAM8 knockout mouse (Kelly, Hutchinson et al. 2005), which suggests ADAM8 as a potential candidate target protein for GBM therapy with minor expected side effects.

In our study, we initially found in GBM tissue that ADAM8 is widely expressed both in neoplastic glioblastoma cells as well as in tumor-associated cells in the tumor microenvironment. We investigated the impact of ADAM8 on GBM progression in vivo by stereotactic injection of ADAM8 deficient glioma cells in the brain of nude mice. Our results suggest a significant contribution of ADAM8 during tumor angiogenesis, as demonstrated in vivo and in vitro by functional analysis of ADAM8 deficient U87 glioma cells and by analyzing primary macrophages isolated from ADAM8 knockout mice.

We found that the angiogenic potential of ADAM8 in GBM cells and in primary macrophages is mediated by the regulation of osteopontin (OPN), an important inducer of tumor angiogenesis. By in vitro cell signaling analyses, we demonstrate that ADAM8 regulates OPN via JAK/STAT3 pathway in U87 cells and in primary macrophages. As ADAM8 is a dispensable protease for physiological homeostasis, we conclude that ADAM8 could be a tractable target to modulate angiogenesis in GBM with minor side-effects.

3.2 Zusammenfassung

Diese kumulative Dissertation fasst zwei Peer-Review-Veröffentlichungen zusammen, die sich mit der Rolle von a Disintegrin and Metalloproteases 8 (ADAM8) in Glioblastoma multiforme (GBM)-Zellen und in der Mikroumgebung von GBM-Tumoren befassen.

Die erste Veröffentlichung beinhaltet den Titel „Molecular profiling of the tumor microenvironment in glioblastoma patients: correlation of microglia/macrophage polarization state with metalloprotease expression profiles and survival “. In dieser Studie untersuchten wir das molekulare Profil von Gliom-assoziierten Makrophagen/Mikroglia (GAMMs) in Korrelation mit der Patientenprognose, indem wir M1/M2-ähnliche Polarisationsmarker in einer Kohorte von 20 GBM-Patienten ausnutzten. Mittels quantitativer PCR (qPCR) wurden die Marker CXCL10 (M1) und CCL13 (M2) in humanen Makrophagen validiert und auf eine globale Analyse von GBM-Gewebe angewendet. Darüber hinaus wurden Proteinase-Gene, die bekanntermaßen mit der GBM-Progression assoziiert sind (ADAM8, MMP9, MMP14, ADAM10, ADAM17), in Korrelation zu M1/M2-Markern analysiert. Unsere Daten zeigten, dass der M2-ähnliche Makrophagenmarker CCL13 signifikant höhere Expressionsniveaus aufwies als alle M1-ähnlichen Makrophagenmarker. Gene für MMP9 und MMP14 sind signifikant mit einem M2-ähnlichen Phänotyp und einer Assoziation mit einer verschlechterten Prognose in der GBM-Patientenkohorte assoziiert. Eine hohe Expression in MMP9 und MMP14 korreliert signifikant ($P < 0,001$) mit einer hohen Expression von ADAM8 in GBM.

Die zweite Publikation mit dem Titel „ADAM8 affects glioblastoma progression by regulating osteopontin-mediated angiogenesis“ untersuchte den funktionellen Einfluss von ADAM8 auf die GBM-Progression in vivo durch stereotaktische Injektion von ADAM8-defizienten Gliomzellen in das Gehirn von Nacktmäusen (nu/nu). Unsere Ergebnisse belegen einen signifikanten Beitrag von ADAM8

während der Tumorangiogenese, wie in vivo und in vitro durch funktionelle Analyse von ADAM8-defizienten U87-Gliomzellen und durch Analyse primärer Makrophagen, die aus Adam8-Knockout-Mäusen isoliert wurden, gezeigt wurde. Bemerkenswerterweise zeigen Mäuse, denen ADAM8 fehlt, keine offensichtlichen Entwicklungs- oder pathologischen Defekte, wie durch den Phänotypisierungsbericht der ADAM8-Knockout-Maus (Kelly, Hutchinson et al. 2005) bestätigt wird. Daher liegt die Annahme nahe, dass ADAM8 ein potenzieller Zielproteinkandidat für die GBM-Therapie mit gering zu erwartenden Nebenwirkungen sein könnte.

In unserer Studie haben wir zunächst in GBM-Gewebe festgestellt, dass ADAM8 sowohl in neoplastischen Glioblastomzellen als auch in tumorassoziierten Zellen in der Tumormikroumgebung weit verbreitet ist. Wir untersuchten den Einfluss von ADAM8 auf die GBM-Progression in vivo durch stereotaktische Injektion von ADAM8-defizienten Gliomzellen in das Gehirn von Nacktmäusen. Unsere Ergebnisse deuten auf einen signifikanten Beitrag von ADAM8 während der Tumorangiogenese hin, wie in vivo und in vitro durch funktionelle Analyse von ADAM8-defizienten U87-Gliomzellen und durch Analyse primärer Makrophagen, die aus ADAM8-Knockout-Mäusen isoliert wurden, gezeigt wurde.

Wir fanden heraus, dass das angiogene Potenzial von ADAM8 in GBM-Zellen und in primären Makrophagen durch die Regulation von Osteopontin (OPN), einem wichtigen Induktor der Tumorangiogenese, vermittelt wird. Durch In-vitro-Zellsignalanalysen zeigen wir, dass ADAM8 OPN über den JAK/STAT3-Weg in U87-Zellen und in primären Makrophagen reguliert. Da ADAM8 eine entbehrliche Protease für die physiologische Homöostase ist, schlussfolgern wir, dass ADAM8 ein handhabbares Ziel sein könnte, um die Angiogenese in GBM mit geringfügigen Nebenwirkungen zu modulieren.

4. Theoretical background

4.1 Glioblastoma multiforme

Glioblastoma multiforme (GBM) is the most aggressive type of brain cancer with a median survival of only 15 months despite aggressive therapy regimens. Until now, the standard clinical treatment of GBM is limited to a combination of surgery, radiation and chemotherapy. Despite continued efforts over several decades to improve patient outcome, GBM remains an incurable disease.

Clinical manifestations of GBM are usually associated with functional correlates of the affected brain regions frontal (25%), temporal (20%), parietal (13%), and occipital (3%)(Blissitt 2014) . Tumors in functional brain areas can provoke obvious symptoms, such as persistent weakness, numbness, decreased vision. Tumors in other regions of the brain may lead to more occult symptoms, such as executive dysfunction, emotional disorders, fatigue, and mild memory disorders. Headache as a preliminary symptom is common and usually associated with significant intracranial hypertension, either directly from the tumor or through obstruction of the ventricular system. Many patients are prescribed corticosteroids at diagnosis to help control vasogenic edema and alleviate accompanying signs and symptoms. A seizure is the presenting symptom in as many as 25% of patients and can occur at a later stage of the disease in as many as 50% of patients(Perry, Zinman et al. 2006, Schiff, Lee et al. 2015)

Computed tomography (CT) or magnetic resonance imaging (MRI) scan are common to use as diagnostic imaging. On MRI, nearly all GBMs enhance with gadolinium contrast and show an irregularly shaped mass with a dense ring of enhancement and hypointense center of necrosis. Necrosis is a hallmark feature of GBM, and presence of necrosis is required for a brain tumor to be grade IV(Blissitt 2014).

Current standard therapy includes surgical resection, followed by concurrent radiation with temozolomide (TMZ) About 70% of GBM patients will experience disease progression within one year of diagnosis with less than 5% of patients surviving five years after diagnosis(Stupp, Mason et al. 2005, Ostrom, Bauchet et al. 2014) Greater extent of resection at recurrence is associated with improved survival(Bloch, Han et al. 2012). Bevacizumab has also been shown to reduce vascular permeability and edema, improve oxygenation, and reduce radiation necrosis. However, it can cause potentially life-threatening events, such as hemorrhage, blood clots, and bowel perforation. It is essential to understand the contribution of the GBM tumor microenvironment. Brain macrophages and microglia particularly contribute to tumor angiogenesis, a major hallmark of GBM.

4.2 Microglia/Macrophage

Microglia are the resident macrophages of the CNS. These mononuclear cells are distributed throughout the brain, where they function as key immune effector cells of the CNS. Using bone-marrow transplantation, investigators concluded that, under homeostatic conditions, a considerable percentage of microglia are replaced by donor-derived monocytes(Biffi, De Palma et al. 2004).

In many neuropathological conditions, the blood brain barrier is impaired, resulting in an infiltration of monocytes from the periphery. Microglia and monocytes converge in high-grade glioma (HGG). HGG cells induce local inflammation that compromises the integrity of the blood-brain barrier (BBB) and results in monocytes infiltrating into the tumor. Once in the CNS, these cells can differentiate into tumor-associated macrophages and become nearly indistinguishable from activated resident microglia.

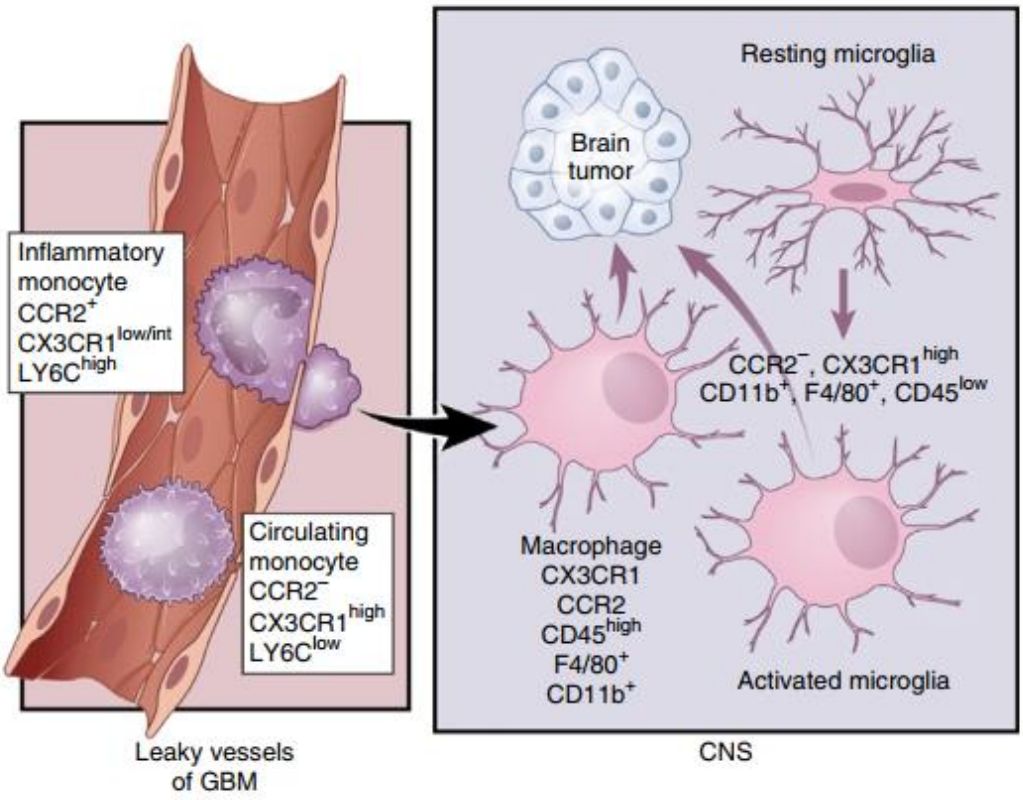

Figure 1. Microglia and monocytes converge in high-grade glioma (HGG) (Cleveland Clinic Center for Medical Art & Photography © 2015)

It is noteworthy that the distinction between pro-inflammatory M1-polarized and anti-inflammatory M2-polarized GAMMs is difficult to study due to the lack of unique markers to assess their activation phenotype in the continuous process of GAM polarization. Nevertheless, M1-polarized GAMs have often been associated with the expression of CD40, CD74, and MHC II, whereas the expression of CD163, CD204, CD206, arginase 1 (ARG1), FIZZ1, and phosphorylated STAT3 (pSTAT3) has been attributed to more M2-polarized GAMs (Orihuela, McPherson et al. 2016). As shown in numerous studies, macrophages can polarize into two extreme types, either M1-like or M2-like. M1-like macrophages are characterized by a pro-inflammatory and anti-tumor phenotype, while M2-like macrophages are considered as anti-inflammatory and tumor-supporting macrophages. According to a previous study performed in GBM patients, ADAM8 expression is high in

macrophages/microglia and equally correlated with M1- and M2-like markers, CXCL10 (M1) and CCL13 (M2)

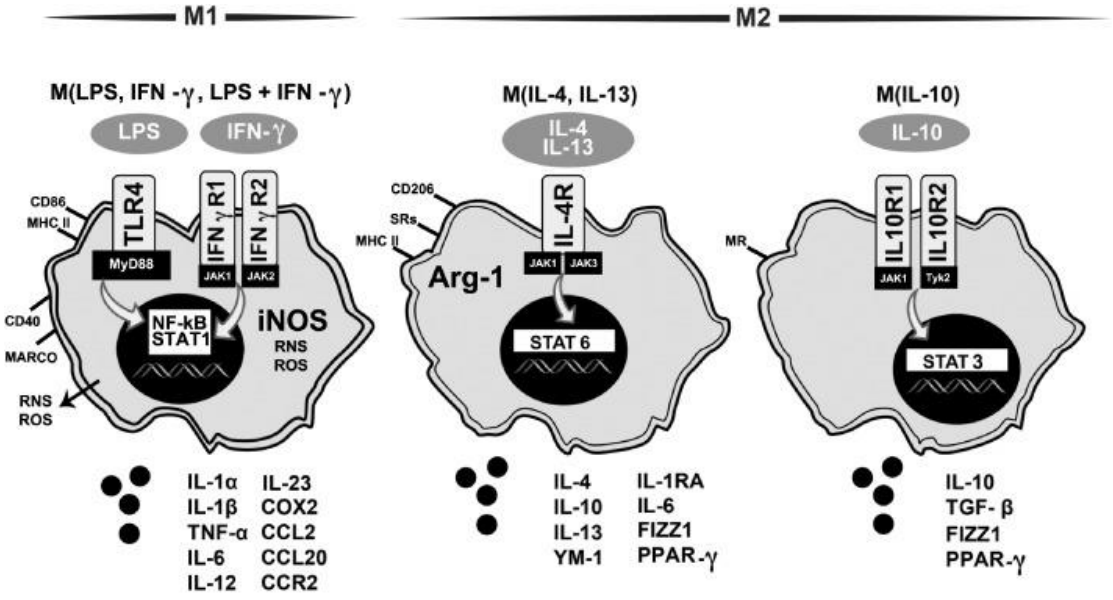

Figure 2 Diagram of activation states of microglia based on inflammatory profile and effector function(Orihuela, McPherson et al. 2016)

Preclinical studies targeting M2-polarized and thus immunosuppressive GAMs revealed promising results(Ieranò, Portella et al. 2016, Mercurio, Ajmone-Cat et al. 2016, Orihuela, McPherson et al. 2016). Therefore, it has been assumed that immunotherapeutic interventions might benefit from additional GAM-directed treatments to improve the prognosis of glioma patients(Poon, Sarkar et al. 2017). There are many drugs developed to target macrophage/microglia.

In 2009, the FDA approved bevacizumab, a drug targeting neovascular formation, for patients with recurrent glioblastomas (Davis 2016). Neo-angiogenesis is critical for tissue growth and repair and forms the basis for tumor progression (Viallard and Larrivee 2017, Javan, Khosrojerdi et al. 2019). Thus, anti-angiogenic drugs have been widely suggested for clinical cancer treatment. This type of treatment is

considered to be beneficial as it can not only reduce the blood and nutrient supply of tumor cells into the tumor core , but also alleviate edema formation and subsequently neurological symptoms (Takano, Kimu et al. 2013, Berghoff, Sax et al. 2014). However, for bevacizumab, fast mutating tumor cells can rapidly develop resistance by circumventing VEGF signaling (Iwamoto, Abrey et al. 2009), so that alternative routes of blocking angiogenesis are required.

As a large non-tumor cell population, macrophages/microglia are prominent cell types of the GBM tumor microenvironment and are mediators of inflammatory processes. Macrophages can build networks in the tumor that form vascular-like structures to provide oxygen and nutrients for tumor growth, thereby promoting tumor growth (Barnett, Rosenfeld et al. 2016).

4.3 A Disintegrin And Metalloproteases (ADAM)

A Disintegrin And Metalloproteases (ADAM) constitute a family of multi-domain enzymes with important roles in cell adhesion, migration, proteolysis and signaling. As transmembrane proteins, ADAMs are associated with the process of ectodomain shedding that releases membrane-associated cytokines and receptors into the tumor microenvironment (Murphy 2008, Kataoka 2009). The metalloprotease-disintegrin 8 (ADAM8), is a proteolytically active member of the ADAM family (Blobel 2005, Murphy 2008) and was initially identified in macrophages (Yoshida, Setoguchi et al. 1990).

ADAM8 (CD156/MS2). Ishikawa et al. screened genes encoding transmembrane/secretory proteins that are upregulated in lung cancers by cDNA microarrays and found that ADAM8 is specifically overexpressed in most cancer tissues and elevated in serum samples from the patients(Ishikawa, Daigo et al. 2004). They also showed that transfection of ADAM8 into tumor cells enhances the invasive activity. Silencing ADAM8 significantly reduced the ability of cell migration

and invasion, and blocked the NF-κB signaling pathway through IκBα and p65 dephosphorylation, leading to reduced NF-κB transcription activity and decreased MMP-13 expression(Liu, Li et al. 2019)

Overexpression of ADAM8 has also been reported in human renal cell carcinomas. Furthermore, ADAM8 is highly regulated in human primary brain tumors such as astrocytomas, and the expression levels and activity are associated with invasiveness(Wildeboer, Naus et al. 2006).These reports suggest that ADAM8 is involved in tumor cell migration and invasion.

High expression levels of ADAM8 in tumor cells have been shown to be associated with invasiveness and metastasis of carcinomas, such as breast and pancreatic tumors (Romagnoli, Mineva et al. 2014, Schlomann, Koller et al. 2015, Conrad, Götte et al. 2017). For these cancers as well as for glioma, high ADAM8 expression levels correlate with poor patient prognosis (Conrad, Benzel et al. 2019).

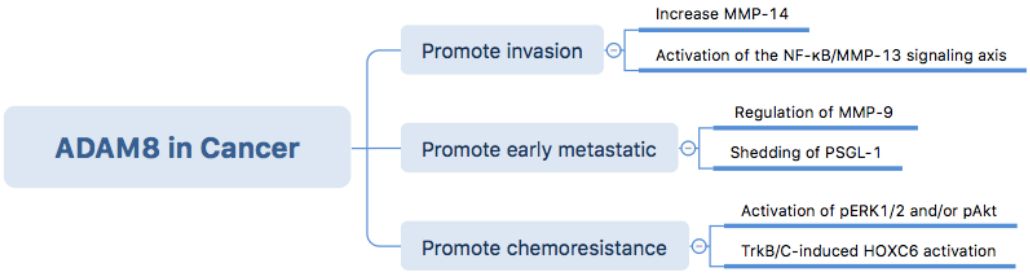

Figure 3. ADAM8 in Cancer

However, the specific contribution of ADAM8 to GBM tumorigenesis remains elusive. Knockdown of ADAM8 in U87 glioma cells led to significantly decreased angiogenesis and tumor volumes of these cells after stereotactic injection into striate body of mice.

We compared the ADAM8 positive and ADAM8KO macrophage supernatant analysis, found that when the ADAM8 is missing, IL-8, Thrombospondin-2, OPN and other cytokines are down-regulated.

4.4 Osteopontin

Osteopontin (OPN) is an extracellular matrix protein also referred to as bone sialoprotein 1 (BSP-1), secreted phosphoprotein 1 (SPP1), and early T lymphocyte activation 1 (ETA-1)(Clemente, Raineri et al. 2016).It is a secreted bone sialoprotein found in all body fluids and expressed in many tissues and cells.

For macrophages, migration of macrophages is influenced by interaction of OPN with α4 and α9 integrins. Moreover, OPN inhibits macrophage apoptosis by interacting with α4 integrin and CD44(Marcondes, Poling et al. 2008). In Dendritic Cells, OPN is expressed at a higher level in immature DCs than in mature DCs; thus, it was suggested that OPN acts as an autocrine and/or paracrine signal for DC maturation(Kawamura, Iyonaga et al. 2005). OPN is also involved in Th cell polarization by enhancing Th1 and Th17 differentiation and inhibiting Th2 cytokine expression. By interacting with CD44 in Th cells, OPN induces hypomethylation of IFN-γ and IL-17α genes enhancing production of IFN-γ and IL-17A.(Guan, Nagarkatti et al. 2009) OPN acts on neutrophil recruitment but has no influence on their phagocytic activity and superoxide, cytokine, and MMP-9 production(Wai and Kuo 2004). In vitro assays showed that the RGD sequence in OPN is required for neutrophil migration and OPN-induced neutrophil migration is dependent on ERK and P38 MAP kinases activation(Atai, Bansal et al. 2011, Hirano, Aziz et al. 2015)

OPN is involved in multiple physiological and pathological processes, starting from inflammation. In particular, OPN plays a key role in cancer progression by enhancing proliferation, survival, motility, and invasion of tumor cells in breast cancer, hepatic carcinoma, prostate cancer, colorectal cancer, lung cancer, and

melanoma(Bandopadhyay, Bulbule et al. 2014) For example, high concentration of OPN cDNA in OPN negative breast cancer cells was shown to promote angiogenesis enhancing tumour progression(Wai and Kuo 2004) .In glioma cells, OPN has been shown to promote proliferation, migration and angiogenesis via integrin αvβ3/PI3K/AKT signaling (Wang, Yan et al. 2011, Ramchandani and Weber 2015). OPN plays a role in the process of angiogenesis due to its high affinity for αvβ3, an integrin highly expressed on particular endothelial cells1. Signalling via αvβ3 is essential for endothelial cell survival, and it has been found that OPN promotes the survival of these cells(Rangaswami, Bulbule et al. 2006)

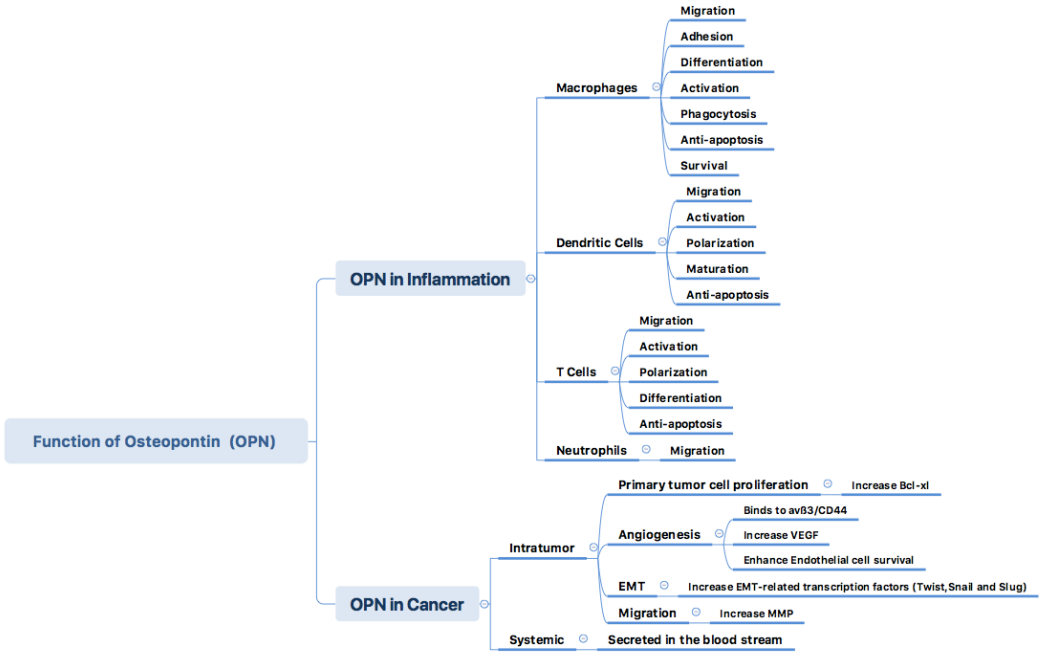

Figure 4. Osteopontin in inflammation and Cancer.

The main role of OPN during inflammation is to trigger different leucocytes eliciting a functional response and inducing cytokine secretion, in order to shape the entire immune response. The OPN overexpression induces multi-steps of cancer cell metastasis through activating different protein mediators. A primary tumour undergoes vascularisation by angiogenesis as various growth factors such vascular endothelial growth factor are secreted. Detachment of the cancerous cell then occurs followed by intravasation; the tumour cell enters and circulates the vascular system. The cell eventually attaches to the wall of the

blood vessel before undergoing extravasation and leaving the blood vessel. The tumour cell then grows as a secondary tumour causing metastasis

4.5 Animal Technology

Mouse macrophages were isolated from mouse bone marrow. Mice were sacrificed by cervical dislocation, and femur and tibia were removed to flush bone marrow out with completed culture medium (10% FCS, 20ng/ml MCSF, DMEM) using a 21G needle. After separating the cells using a cell strainer (pore size 100µm) , cells were seeded in a petri dish in 10ml complete medium. At day 4, 5 ml of the complete medium were replaced. After 7 days of incubation, macrophages were used for functional experiments.

5. Summary of the publications

5.1 Molecular profiling of the tumor microenvironment in glioblastoma patients: correlation of microglia/macrophage polarization state with metalloprotease expression profiles and survival

Marko Gjorgjevski*, Ricarda Hannen,*, Barbara Carl, **Yu Li**, Emilie Landmann, Malte Buchholz, Jörg W. Bartsch and Christopher Nimsky: Molecular profiling of the tumor microenvironment in glioblastoma patients: correlation of microglia/macrophage polarization state with metalloprotease expression profiles and survival.(*shared first authors) Biosci Rep. 2019 Jun 20;39(6):BSR20182361. doi: 10.1042/BSR20182361.

Impact factor:2.942 (2020)

5.1.1 Validation of macrophage markers in polarized THP1 cells and blood derived PBMCs

To test the validity of qPCR markers for macrophage phenotype analysis, we used THP-1 cells and PBMCs as two independent macrophage-like cell types for our analyses.

THP-1 cells as monocytic cells derived from acute monocytic leukemia were polarized in vitro and expression level of polarized cells was compared to M0 phenotype. All four analyzed M1 macrophage markers (CXCL9, CXCL10, IL12B, and CD38) showed a good discrimination depending on the polarization state, although for IL12B the discrepancy was not as high as for CXCL9, CXCL10, and CD38. In contrast, the tested M2 macrophage markers (CCL13, EGR2, and

CD206) were more heterogeneous in expression. CCL13 and CD206 expression best reflected the M2-like macrophage polarization state. In addition, M1/M2 markers were tested in polarized PBMCs.

In PBMC derived macrophages all four M1 markers show high expression levels only in M1 polarized macrophages; in particular, CXCL10 showed the highest difference when comparing M1 with M2 polarized macrophages. For M2 markers tested, the results were more heterogeneous. Whereas EGR2 and CD206 markers showed no difference comparing M1 with M2 polarization, only CCL13 was identified as the most distinct M2 marker.

From these experiments we conclude that CXCL10 and CCL13 are the most informative markers to discriminate between M1- and M2-like macrophage populations, respectively, given their expression levels in THP1 and PBMC derived macrophages. Therefore, these two markers were used in the following experiments with the aim to describe the global microglia/macrophage polarization state in GBM.

Given these validated macrophage polarization markers, we also analyzed the differences in protease expression levels in correlation to M1/M2 polarization in PBMCs. Protease genes were selected by their reported involvement in GBM pathology and their association with patient survival as analyzed in the global gene expression database TCGA. TCGA data analysis of 159 GBM patients revealed either an association with no significant effects on overall survival for ADAM10 (384 d for high vs. 432 d for low expression, P = 0.6036) and for ADAM17 (442 d for high vs. 405 d for low, P = 0.5412). Moreover, proteinases ADAM8 (360 d for high vs. 454 d for low, P= 0.0685) and MMP14 (375 d high vs. 460 d low, P = 0.0824) are significantly associated with an impaired patient prognosis when highly expressed. A similar tendency, although not statistically significant, was observed for MMP9 (360 d for high vs. 468 d for low, P = 0.2371). Thus, we hypothesized that

proteinase gene expression might be clustered with a distinct microglia/macrophage phenotype. M1-like macrophages compared to M2-like macrophages show higher expression levels for all protease genes analyzed: MMP9, MMP14, ADAM8, ADAM10, and ADAM17. The expression levels of MMP genes were generally higher than expression of ADAM protease genes.

5.1.2 An M2-like microglia/macrophage subtype is predominantly expressed in GBM

The macrophage markers described above were used to analyze macrophage subtypes in GBM tissue in a patient cohort of 20 patients. The M2 macrophage marker CCL13 showed significantly higher expression levels than all M1 macrophage markers. This supports the notion that the M2-like macrophage phenotype is the predominant one in GBM.

To further investigate a possible link between macrophage phenotype and protease expression, qPCR analysis for expression levels of MMP9, MMP14, ADAM8, ADAM10, and ADAM17 were performed. The results show relevant mRNA expression levels for all proteases investigated.

5.1.3 ADAM10 and ADAM17 expression linked to M1-like, MMP9 and MMP14 expression linked to M2-like microglia/macrophage

Since the goal of this study was to provide clues on expression levels of proteases to a specific microglia/macrophage polarization type, macrophage marker expression was correlated to protease expression. Our findings indicate that ADAM10 and ADAM17 are mainly associated with M1-like microglia/macrophage, as both proteases correlated significantly (for ADAM10 $P<0.01$ and for ADAM17 $P<0.05$) with the M1 macrophage marker CXCL10. In contrast, MMP9 and MMP14 seem to be expressed by M2-like microglia/macrophage demonstrated by a significant (for MMP9 $P<0.05$ and for MMP14 $P<0.001$) linkage to CCL13 expression.

We know from the experiments above that the expression of M2 in GBM is dominant, and there are differences in the expression of M1 and M2 macrophages at the protein level, among which there are obvious differences. Consequently, we constructed an *in vitro* macrophage model to study the effect of ADAM8 existence on tumor behavior.

5.2 ADAM8 affects glioblastoma progression by regulating osteopontin-mediated angiogenesis

Yu Li, Songbo Guo, Kai Zhao, Catharina Conrad, Caroline Driescher, Vanessa Rothbart, Uwe Schlomann, Helena Guerreiro, Miriam H. Bopp, Alexander König, Barbara Carl, Axel Pagenstecher, Christopher Nimsky and Jörg W. Bartsch : ADAM8 affects glioblastoma progression by regulating osteopontin-mediated angiogenesis Biol Chem. 2020 Sep 11:/j/bchm.ahead-of-print/hsz-2020-0184/hsz-2020-0184.xml. doi: 10.1515/hsz-2020-0184.

Impact factor:5.486 (2022)

5.2.1 ADAM8 expression is increased in Glioblastoma multiforme and is localized to tumor cells and tumor-associated macrophage/microglia

A total of 50 GBM tissues obtained from hospitalized patients were examined for ADAM8 expression by qPCR, indicating that ADAM8 expression is significantly higher in GBM compared to normal brain tissue. Western Blot analyses confirmed higher expression levels of ADAM8. Furthermore, ADAM8 localization in GBM tissue was assessed by immunohistochemistry. In normal brain tissue, ADAM8 expression is hardly detectable and restricted to some neuronal cells. In GBM tissue, ADAM8 expression is significantly higher (approx. 10-fold, $p<0.05$) and can be detected in tumor cells, neoplastic endothelial cell with proliferations, and in tumor-associated cells. In order to characterize these cells further, we performed double staining with immune cell markers and found that ADAM8 co-localizes with CD68, a marker for macrophages/microglia in the brain. Thus, we conclude that ADAM8 might be functionally important for tumor cells and for tumor-associated macrophages.

To analyze the function of ADAM8 in GBM, we performed loss-of-function experiments in U87MG cells. Among all human GBM cell lines, U87MG cells showed the highest level of ADAM8 expression. Briefly, endogenous ADAM8 was knocked down by small hairpin loop RNAs (shRNA) and a number of cell clones (n=8) were obtained with significantly reduced ADAM8 expression levels (>50-fold). The cell clone with the highest ADAM8 reduction assessed by qPCR and western blot was used for further functional analyses in vitro and in vivo. The proliferation behavior of cells showed no difference, while cell migration was significantly reduced in U87_A8ko cells.

5.2.2 ADAM8 reduced angiogenesis and tumor volume in a GBM model

Using U87 control (scramble, U87_shCtrl) and U87 ADAM8knockdown (U87_shA8) cell clones, intracranial tumor cell injections into striatum of nude mice were carried out to assess tumorigenesis. To detect tumors, MRI scanning was started 3 weeks after implantation in weekly intervals and tumor volumes were calculated (n=14 for each group). All mice injected with U87_A8ko cells survived over an observation time of nine weeks. In contrast, all mice injected with U87_shCtrl cells reached the endpoint criteria or died spontaneously within the observation time. Compared to the U87 control group, tumor volumes of U87_A8kd cells were significantly smaller and, in some cases (6 out of 14), tumor cells were completely absent 3 weeks after implantation. To assess a possible tumor graft rejection, injection sites were analyzed 1 week after injection. Whereas tumors derived from U87_shCtrl cells grew as a compact cell mass after 1 week, U87_A8kd cells showed a patchy growth characteristic. After staining for vascular structures using CD31 staining, we found significantly less (reduced to 46 %, $p<0.01$) vascular staining in U87_shA8 (8.7x 104 ± 8) compared to the U87_shCtrl group.

5.2.3 ADAM8 regulates GBM cell angiogenesis via osteopontin

Given the results of the in vivo experiments, we postulated that U87_shA8 cells could be deficient in angiogenesis induction. To test this hypothesis in vitro, we utilized conditioned medium from U87_shCtrl and U87_shA8 cells for angiogenesis induction of Human Umbilical Vein endothelial cells (HUVEC) and quantified formation of tubes. After 6 hours incubation with the respective cell culture supernatants, tube formation was quantified by counting the number of meshes formed. ADAM8 containing supernatants from U87_shCtrl cells induced significantly higher numbers of tubes as compared to supernatants from ADAM8-deficient U87_shA8 cells ($P<0.05$).

To identify the molecular mechanism by which ADAM8 affects angiogenesis, a differential analysis of cell supernatants from U87_shCtrl and U87_shA8 cells was conducted in an Angiogenesis Protein Profiler Array. Array membranes with spotted antibodies against proteins involved in angiogenesis were incubated with conditioned supernatants of U87_shCtrl and U87_shA8 cells and the proteins captured by antibodies on the array membrane were detected by chemiluminescence and quantified. Several candidates were identified, most prominently IL-8 and Osteopontin (OPN).

Differences in expression levels of OPN between U87_shCtrl and U87_shA8 cells was confirmed by qPCR, suggesting that OPN expression levels are dependent on the ADAM8 gene dosage.

To provide a direct proof for our hypothesis, we analyzed whether exogenous OPN can be used to recover the deficiency of U87_shA8 cells in angiogenesis assays. Different concentrations of OPN were added to U87 supernatants in angiogenesis assays. When adding IL-8, the reduced angiogenesis capacity of U87_shA8 cells was not restored. In contrast, addition of recombinant OPN at different concentrations caused an increase in the number of meshes formed in the U87_shA group and reached similar values as in the U87_shCtrl group. As the OPN

concentration increases, the number of meshes increases in both groups, although to a smaller extent in U87_shCtrl cells due to saturation effects.

5.2.4 ADAM8 affects secretion of OPN via the JAK/STAT3 pathway in GBM cells

From previous studies it is known that ADAM8 can directly affect intracellular signaling by either binding to integrin β1 or by direct interaction with intracellular proteins interacting with the ADAM8 cytoplasmic domain. To explore the signaling pathway involved in OPN regulation by ADAM8, we used a panel of pathway inhibitors to target NF-kB, JAK/STAT3, p38, ERK1/2 and AKT/PI3K. U87_shCtrl and U87_shA8 cells were incubated with the respective inhibitors for 24 hours and the levels of secreted OPN in cell supernatants were determined by ELISA. While none of the inhibitors affects OPN secretion in U87_shA8 cells, OPN secretion was significantly decreased in U87_shCtrl cells treated with the JAK/STAT3 inhibitor only, whereas other inhibitors such as NF-κB and p38 inhibitor caused even slightly enhancing effects on OPN secretion.

5.2.5 In macrophages, ADAM8 is involved in the regulation of angiogenesis via OPN

As shown above, ADAM8 is additionally expressed in GBM associated macrophages/microglia (GAMMs) [9] which contribute to tumor angiogenesis in GBM. To analyze if ADAM8 affects OPN expression in these cells similar to tumor cells, we used primary macrophages isolated from wild-type (WT) and Adam8-deficient mice (Kelly, Hutchinson et al. 2005) and no difference in macrophage development was observed. Primary macrophages were isolated from bone marrow with a purity of >90%. Supernatants from macrophages were collected and angiogenesis assays using HUVEC cells were performed. After 6 hours incubation with supernatants from WT and Adam8-deficient macrophages, HUVEC tube formation was significantly lower in supernatants from Adam8-deficient

macrophages compared to wild-type macrophages. Similar to U87 cells, we analyzed differential proteins secreted from macrophages using a cytokine array. The strongest difference between WT and Adam8-deficient macrophages was observed for osteopontin.

5.2.6 ADAM8 regulates OPN via JAK/STAT3 and NF-κB in macrophages

Expression levels of OPN in WT and A8KO macrophages were confirmed by qPCR and ELISA assays. We further analyzed if OPN added to A8KO macrophages restores angiogenesis; 100ngl OPN restores the decrease of angiogenic ability caused by ADAM8 deletion but had little effect on WT macrophage supernatant. Signaling pathway inhibitors were used in macrophages. Protein levels of OPN in cell supernatants were determined by ELISA. Inhibition of JAK/STAT3 and NF-κB caused a significant reduction of OPN expression in WT and A8KO macrophages, suggesting that ADAM8 is one of the upstream proteins to regulate OPN expression through JAK/STAT3 and NF-κB.

6. Discussion

We demonstrate that the tumor microenvironment in GBM patient cohort can be profiled by M1-like and M2-like microglia/ macrophage markers CXCL10 (M1-like) and CCL13 (M2-like) and by a subset of metalloprotease genes. Our findings on GBM tumors are supported by the correlation of M1/M2 markers with patient survival and support the notion that, although not statistically significant in our limited patient cohort, a predominant M2-like microglia/macrophage polarization is associated with an impaired prognosis whereas a predominant M1 polarization is associated with better overall prognosis of GBM patients. These results were obtained from a patient cohort of 20 well-documented GBM patients so that a larger patient cohort could lead to a higher significance of our results. In previous studies, a microglia/macrophage polarization type distinct from M1/M2 was reported to be associated with GBM. However, these microglia/macrophage polarization data are derived from GL261 injected C57BL/6 mice (Szulzewsky, Pelz et al. 2015) and might not be transferable to human GBM.

Expression of ADAM8 has been described to be involved in tumor cell invasion in gliomas (Wildeboer, Naus et al. 2006) and high expression levels are unfavorable for glioma patients (He, Ding et al. 2012). More recently, ADAM8 expression was correlated to the occurrence of glioma-associated macrophages/microglia (GAMMs, (Gjorgjevski, Hannen et al. 2019), however the function of ADAM8 in tumor cells and in GAMMs remains elusive and tumor promoting effects in the tumor microenvironment can be postulated, since in all tumor entities described so far, ADAM8 is responsible for tumor invasion, migration, and chemoresistance, leading to poor clinical outcomes (Conrad, Benzel et al. 2019).

We found that GBM tissues investigated from our own tumor bank (n=50 patients) show elevated expression of ADAM8. Furthermore, the presence of ADAM8 in GBM is mainly associated with increased angiogenesis. To analyze if angiogenesis induction is based on a systemic effect, we investigated tumor cells and macrophages separately *in vitro* using genetic means to reduce the *ADAM8* gene dosage. Our results show that ADAM8 induced in GBM can either be expressed in tumor cells or in GAMMs. As demonstrated previously, there is no association of ADAM8 expression with a certain macrophage polarization state (M1/M2), so we can conclude that potentially both macrophage states could contribute to the observed angiogenesis effect (Dai, Peng et al. 2009).

As active metalloprotease, ADAM8 can cleave substrates relevant for angiogenesis, as shown in the context of retinal neovascularization (Guaiquil, Swendeman et al. 2010). However, in this experimental setting, ADAM8 as a sheddase of angiogenesis factors such as Tie-2, VE-cadherin, and CD31 has an opposite effect on angiogenesis, i.e. blocks angiogenesis by inactivating angiogenesis-related proteins. By investigating the release of angiogenesis related molecules, we noticed that osteopontin is regulated by ADAM8 at the transcriptional level, leading to decreased levels of both, RNA and secreted OPN in tumor cells and macrophages as a result of reduced ADAM8 levels. Thus, ADAM8, via a non-proteolytic function, can modulate intracellular signaling in both cell types, thereby affecting tumor angiogenesis.

Further we confirm that OPN could indeed promote the angiogenesis ability of HUVEC cells, we performed OPN "rescue" assays. When adding OPN to U87 cell supernatant of ADAM8 knockdown cells, OPN restores the ability of U87 cells to promote angiogenesis. In a study investigating the functional effect of OPN on HUVEC cells (Dai, Peng et al. 2009), a concentration of up to 5μM (44 μg/ml) was used to activate PI3K/AKT and ERK1/2 signaling pathways. In our study, we used

up to 200 ng of OPN corresponding to 0.2 μM, a concentration that is highly potent in inducing angiogenesis. For wild-type U87 supernatants, tube formation was only slightly increased compared to wild-type U87 supernatants without OPN. This indicates that at this point the ability of tube formation of HUVECs reached saturation. In contrast, when OPN was added to U87_shA8 cell supernatants, the ability of tube formation reached similar levels than the angiogenesis potential of U87_shCtrl cells. This prompted us to conclude that OPN is an ADAM8-dependent factor essential for HUVEC tube formation and for tumor angiogenesis. In this regard, there are numerous studies supporting the notion that OPN promotes tumor angiogenesis. OPN was found along with VEGF in leukemia, involved in angiogenesis and chemoresistance (Mirzaei, Mohammadi et al. 2018). In primitive connective tissue, OPN contributes as a key element to enhance osteogenesis and angiogenesis (Carvalho, Silva et al. 2020). In endothelial cells, OPN stimulates angiogenesis via phosphorylation and activation of the PI3K/AKT and ERK1/2 pathway (Ramchandani and Weber 2015). In conclusion, OPN has a general effect on angiogenesis promotion on different types of cells and expression of OPN in GAMMs is particularly essential for angiogenesis in GBM (Guaiquil, Swendeman et al. 2010). In addition, OPN is essential for infiltration of GAMMs in GBM (Wei, Marisetty et al. 2019). In GBM patients, elevated OPN levels were detected in serum samples, and their levels correlate with poor prognosis (Sreekanthreddy, Srinivasan et al. 2010).

Our study provides novel evidence that ADAM8 regulates OPN expression and secretion. In order to further understand the mechanism of this regulation, several signal pathway inhibitors were used to block the specific pathways of NF-κB (Berbamine dihydrochloride), JAK/STAT3 (WP1066), P38 (SB203580), ERK1/2 (U0126), and PI3K/AKT (LY294002). We found that predominantly blocking JAK/STAT3 can significantly reduce expression levels of OPN, while other pathway inhibitors have an opposite (NF-κB) or no effect (p38, ERK1/2, PI3K/AKT) on OPN

release in U87_shctrl, but not in U87_shA8 cells. Based on these findings, we can conclude that ADAM8 derived from U87 can promote angiogenesis through OPN via the JAK/STAT3 pathway. STAT3 in particular was described as an important factor for glioma infiltration and growth (Priester, Copanaki et al. 2013) and the activation of this pathway by ADAM8 presents a novel finding.

Interestingly, a similar regulation of OPN by ADAM8 was also found in bone-marrow derived macrophages. To analyze this, we used primary macrophages from *Adam8*-deficient mice as the most suitable cell model. In these macrophages, not selected for a certain polarization type, expression of OPN decreased in the absence of ADAM8, so that the ability to stimulate angiogenesis *in vitro* decreased accordingly. Analogous to the experiments with U87 cells, exogenous addition of OPN restores the observed decrease of angiogenesis caused by ADAM8 deficiency, demonstrating that an ADAM8 to OPN signaling cascade is mode of action in these cell types with regard to angiogenesis. Considering macrophages as an eminent cell type in the microenvironment of GBM, elimination of ADAM8 in the tumor microenvironment could cause deprivation of OPN, thereby reducing neovascularization of the tumor blood supply. In contrast to U87 cells, OPN secretion in WT and in *Adam8*-deficient macrophages can be reduced by the JAK/STAT3 inhibitor. This suggests that there is an ADAM8-dependent and -independent signaling for JAK/STAT3 activation in macrophages. However, the ADAM8-independent OPN regulation occurs almost at a very basal level of expression. In macrophages, there is an additional effect of NF-κB inhibition on OPN expression.

So far it was unknown how ADAM8 affects OPN expression and secretion. We have seen that the effect of ADAM8 on OPN is independent of its metalloprotease function. Rather, the disintegrin domain and signaling via the cytoplasmic domain seems to be involved in OPN regulation. A few clues came from previous research.

In our previous studies of pancreatic cancer, we found that the expression of ADAM8 in pancreatic cancer cell lines Panc1 and AsPC-1 is related to migration and apoptosis. Invasiveness of pancreatic ductal carcinoma induced by activation of PI3K/AKT and ERK1/2 suggested that ADAM8 is highly correlated with PI3K/AKT and ERK1/2 signaling pathways (Schlomann, Koller et al. 2015). A previous study found that ADAM8 promotes migration and invasion of chondrosarcomas by activating NF-κB/MMP-13 (Liu, Li et al. 2019). JAK/STAT3 has been proven to be related to angiogenesis in some studies (Valdembri, Serini et al. 2002, Barry, Townsend et al. 2007, Xue, Xie et al. 2017). By ELISA analysis, we found that the amount of secreted OPN in macrophages stimulated by JAK/STAT3 and NF-κB inhibitors decreased significantly, suggesting that OPN is regulated by these pathways.

In summary, our study establishes M1-/M2-like markers CXCL10 and CCL13 for informative and reliable detection of GBM associated microglia/macrophage polarization in conjunction with a defined protease profile as molecular determinants for GBM progression. These findings can be converted into a diagnostic mean to predict patient prognosis, therapy response, and could aid to define those patients for which reprogramming of GAMs could be beneficial.

In addition, we identified ADAM8 as a relevant metalloprotease-disintegrin in GBM. Since ADAM8 is present in tumor cells and in GAMMs, we hypothesize that ADAM8 is an important modulator of the tumor microenvironment. As the major target protein of ADAM8, we identified OPN that is regulated by ADAM8 via JAK/STAT3 signaling in GBM cells and in macrophages, thereby explaining the role of ADAM8 in neovascular formation. Further studies will proof these mechanisms in an animal model and further exploit these mechanisms *in vivo* with the goal to establish ADAM8 as a drug target in glioblastoma.

7. Literature

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8. Appendix

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8.2 Declaration

"I declare on my honor that I have submitted the work to the Medical Faculty Marburg for the doctoral examination with the title Dr. med. in the laboratory of the Department of Neurosurgery under supervision of Prof. Jörg W. Bartsch. I did it myself without any help and did not use any aids other than those listed in the dissertation when writing the thesis. So far, I have not submitted an application for admission to a doctorate at any domestic or foreign medical department, nor have I submitted this or any other work as a dissertation.

I assure you that I have marked all literal or analogous adoptions and quotations.

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