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DISSERTATION

The Influence of Host-Derived Osteopontin on Glioma

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1 Abstract

Glioblastoma multiforme (WHO Grade IV) is the most common malignant neoplasm of the brain. Hallmarks of WHO grade IV designated tumors are a rapid pre- and postoperative disease evolution and a fatal outcome. Those features are also reflected in the low overall survival rate of GBM patients of under 12 months, despite adjuvant radio-chemotherapy.

Research has not just been focusing on the actual neoplastic cell mass but also on the tumor microenvironment. Microglia and peripheral macrophages/monocytes can account for up to 45% of the total brain-tumor mass. Interacting with cancer cells they do not seem to display anti-tumoral functions. In contrary they appear to support tumor growth by degrading the extracellular matrix as well as by secreting immunosuppressive factors. Recent findings reveal that glioma associated microglia (GAMS) display a distinctive gene expression profile compared to the classical M1 M2a, M2b and M2c phenotypes. One gene already investigated in various cancer entities including glioma, was shown to be amongst the highest upregulated genes in the set of GAMs, namely *Spp1* encoding for osteopontin.

Those data were affirmed by q-PCR, identifying GAMs as the main source of osteopontin transcripts both in mouse and in human. Glioma-cell derived osteopontin is known to enhance migration, invasion, and stem cell like character as well as radiation resistance in glioma. So far there has been no data investigating the influence of microenvironmental osteopontin on glioma growth and progression. We used a GL261 model in OPN-/- mice to measure disease progression and properties of the tumor microenvironment in the absence of osteopontin. Our results are based on westernblot, quantitative reverse transcriptase PCR, immunohistochemistry and FACS-analysis. Our findings show that the loss of host-derived osteopontin leads to a faster disease progression and accelerated tumor growth. Another observation was that OPN-/- mice had a reduced number of microvessels and less pericytes surrounding tumor-vessels. Furthermore, there was a change in the composure of the tumor microenvironment between OPN-/- mice and wild type control mice, showing an increase in microglia population in OPN-/- mice. There was no difference in the cytokine profile of OPN-/- and wild-type GAMs. Another result was that in comparison to cultured wild-type microglia, GAMs solely express the secreted and not the intracellular form of osteopontin. This study shows for the first time that host-derived osteopontin seems to negatively influence tumor growth. Furthermore, it seems to render the integrity of tumor vasculature and the composure of tumor-infiltrating immune cells.

2 Abstract in German

Das Glioblastom ist der häufigste maligne Tumor des Gehirns. Als WHO Grad IV Tumor zeichnet sich dieser Hirntumor durch einen schnellen prä- und postoperativen Progress sowie seinen fatalen Endpunkt aus. Das spiegelt sich in der niedrigen Gesamtüberlebensrate von Glioblastompatienten von unter 12 Monaten trotz adjuvanter Radio-Chemotherapie wieder. Die Forschung hat sich nicht nur mit der eigentlichen neoplastischen Zellmasse sondern auch mit der Tumorumgebung beschäftigt. Mikroglia/Makrophagen können bis zu 45% der Gesamttumormasse ausmachen. Agieren diese mit den Tumorzellen, scheinen sie keine Eigenschaften im Sinn einer Tumorabwehr zu besitzen. Im Gegenteil erscheint es, als würden sie das Tumorwachstum unter anderem durch ein Zersetzen der Extrazellulären Matrix sowie durch die Sekretion immunsuppressiver Faktoren unterstützen. Kürzlich durchgeführte Studien zeigen, dass diese so genannten Gliom-assoziierten Mikroglia/Makrophagen (GAMs) ein, von den klassichen M1, M2a, M2b und M2c-Phänotypen, abweichendes Genexpressionsmuster zeigen. Ein Gen, welches bereits im Kontext verschiedener Tumor-Entitäten, darunter dem Glioblastom, untersucht wurde, zeigte sich im Vergleich zu den charakteristischen Phänotypen unter den am höchsten exprimierten Genen, nämlich *Spp1*, welches für Osteopontin kodiert. Diese Daten wurden durch q-PCR verifiziert und identifizieren GAMs als die Hauptquelle von transkribiertem Osteopontin sowohl in der Maus als auch in humanen Proben. Es ist bekannt, dass Osteopontin aus Gliomzellen die Migration, die Invasion, den Stammzellcharakter sowie die Strahlenresistenz des Tumors verstärkt. Bisher gibt es keine Daten, welche den Einfluss auf das Tumorwachstum und den Tumorprogress von Osteopontin, welches der Tumorumgebung entstammt, untersuchen. Wir haben ein GL261 Modell in OPN-/- Mäusen benutzt, um den Tumorprogress und die Eigenschaften der Tumorumgebung in Abwesenheit von Osteopontin zu erforschen. Unserer Ergebnisse basieren auf Westernblots, quantitativer reverser Transkriptase PCR, Immunhistochemie und FACS-Analysen. Unsere Resultate zeigen, dass der Verlust des dem Wirt entstammenden Osteopontins zu einer schnelleren Krankheitsprogression und einem beschleunigten Tumorwachstum führt. Eine weitere Beobachtung war, dass OPN-/- Mäuse eine reduzierte Anzahl an Mikrogefäßen und weniger, die Tumorgefäße umgebenden, Perizyten aufwiesen. Zudem zeigte sich ein Unterschied bezüglich der Zellzusammensetzung der Tumorumgebung zwischen Wildtypmäusen und OPN-/- Mäusen, welcher einen Anstieg der Microglia-Population in OPN-/- Mäusen zeigte. Es ergab sich kein Unterschied bezüglich des Zytokinprofils zwischen Wildtyp-und OPN-/- Mäusen. Ein weiteres Ergebnis war, dass GAMs, verglichen mit kultivierten Wild-Typ-Mikroglia nur die sekretierte und nicht die intrazelluläre Form von Osteopontin exprimieren. Diese Arbeit zeigt zum ersten Mal, dass dem Wirt

entstammendes Osteopontin das Tumorwachstum negativ beeinflusst. Zudem scheint es die Integrität der Tumorgefäße und die Zusammensetzung der den Tumor infiltrierenden Immunzellen zu verändern.

3 Synopsis

3.1 Introduction

3.1.1 Primary Brain and CNS Tumors

3.1.1.1 Epidemiology

Gliomas, according to the Central Brain Tumor Registry of the United States (CBTRUS) Statistical Report, account for 28% of all Primary Brain and CNS tumors, and within those for 80% of the malignant tumors. Glioblastoma multiforme (GBM) is with 45.6% the most common primary malignant neoplasm of the brain. The median age at diagnosis is between 64 and 65, whereas the incidence rate increases with age and is highest among the 74- to 84-year-old's[1].

3.1.1.2 Histological and Molecular Classification Systems

In 1979 the first edition of the World Health Organization (WHO) Classification of Tumors of the Central Nervous System titled *Histological Typing of Tumors of the Central nervous system* was published. It soon became the internationally recognized standard work for the characterization of CNS tumors. In order to meet the demands regarding a diagnostically conclusive classification system, histological as well as clinical malignancy were taken into account. The result was a four grades comprising scheme (WHO I-IV) including cell origin, mitosis, vascular proliferation, cellularity, atypia and necrosis as well as prognosis and recurrence.[2] In the following decades the WHO grading system underwent three updates. All of them are based on the histological way of grading. 2016 World Health Organization Classification of Tumors of the Central Nervous System incorporated molecular features for the first time in order to characterize CNS tumor entities. This leads to an integrated classification system being based on histological as well as genotypic parameters. Although the genotype is prioritized over the phenotype in case of inconsistency, histological classification is still essential for defining the WHO grade.

3.1.1.3 Diagnosis and Current Standard of Care

Current standard of care for WHO grade IV gliomas, depending on lesion side and proximity to eloquent areas, includes maximal surgical resection or biopsy and adjuvant radiochemotherapy consisting of temozolomide (TMZ) and a radiation dose of 60 Gy administered in 30 fractions. This is followed by another six cycles of temozolomide, which is an alkylating agent. Information based on the NOA-8 and the Nordic Trial, two randomized controlled studies, suggests that a therapy with TMZ only is not inferior to a therapy comprising TMZ and radiotherapy (RT) for patients 60 years and older with concomitant MGMT promotor methylation. Furthermore, there is evidence that patients lacking an MGMT-promotor methylation show an enhanced event-free survival being administered to RT only as compared

to the concomitant TMZ arm. Regarding anaplastic oligodendroglioma there is evidence that adjuvant RT in combination with procarbazine, lomustine and vincristine (PVC) leads to prolonged survival compared to RT alone. [3, 4] There is new evidence that in patients with low-grade gliomas (WHO I-II) the RT+ PVC- arm shows increased overall survival rates compared to patients with RT only. Another study comparing the treatment effect in low grade gliomas of RT vs RT and TMZ shows a significant survival benefit only in patients with IDH mutation but not so in IDH wildtype or 1p/19q co-deleted tumors.[5]

3.1.1.4 The Tumor Microenvironment and Immunotherapeutic Approaches

Despite multimodal treatment, high-grade gliomas remain to be associated with a very poor prognosis and treatment options are still limited. For that reason, new approaches focusing not only on the actual neoplastic-cell-mass but also on tumor microenvironment and the resultant diversity of interactions were adopted.

Various types of non-transformed cells compose the glioma tumor-mass. This includes resident cells of the brain like astrocytes, microglia, endothelial cells and pericytes as well as peripheral immune cells and cells with stem-cell-like character. Cells with immune effector function thereby seem to be able to harbor anti- as well a pro-tumorigenic functions. [6] This is due to the fact that tumor cells undergo immunoediting, a process driven by selection pressure exerted by immune surveillance that results in a diminished immunogenicity and thereby in immune escape [7]. Further it enables tumor cells to manipulate immune cells and tissue resident cells to promote tumor-growth [8].

Astrocytes, accounting for up to 50% of the cell population of a healthy brain, physiologically become activated by CNS-injury and form a cell-layer around the damage. This process is called reactive gliosis and results in the upregulation of inflammatory cytokines, growth factors and extra-cellular matrix proteins. It is designed to curb and repair brain damage, but in the context of glioma it facilitates glioma progression through various mechanisms [9]. By secreting factors like IL-6 and TGF-ß astrocytes accelerate glioma proliferation [10, 11]. They also support glioma invasion by cleaving pro-MMP-2 and thereby converting it into its functional extra-cellular-matrix degrading form [12]. Astrocytic connexin43, a major gap junction protein, is enhanced in glioma and is also connected to an elevation of glioma invasiveness [13]. Furthermore, it has been suggested that astrocytes exert chemoprotection over glioma cells by upregulating a set of survival genes via gap junctional communication, thereby attenuating the effect of chemotherapeutic drugs like temozolomide [14].

Gliomas are also highly vascularized tumors and therefore create a special environment for a multitude of cells within the tumor vasculature. That vasculature results from different mechanisms of neovascularization. While initially glioma cells grow around pre-existing

vessels, coopting them in the process, angiogenesis is triggered later in tumor progression by the release of hypoxia-inducible factors and is defined by the sprouting of vessels of already existing vasculature. Vasculogenesis on the other hand is driven by the recruitment of endothelial progenitor cells, thus leading to a de-novo-formation of vasculature. The created perivascular niche is responsible for maintaining a cell population termed glioma stem cells (GSCs). Those Cd133+ cells have the abilities of self-renewal and differentiation as well as oncogenesis. They utilize the proximity to blood vessels and secrete amongst others VEGF, thus further spurring angiogenesis and upholding their own environmental demands. Above that GSCs are also capable of transdifferentiating into endothelial like-cells. This is giving rise to an alternative VEGF-independent microvasculature formation [15-17]. GSC are also partially responsible for tumor-recurrence and chemotherapeutic-resistance [18, 19].

Lymphoid cells in glioma, comprise mainly of T-cells but also of B-cells and NK-cells and account for only around 2.48% of the total cell population [20]. While a high fraction of CD8+ cytotoxic T-lymphocytes is associated with a more beneficial outcome [21, 22], CD4+ T-cells have to be further segregated into Th1, Th2, Th17 and Treg subsets [23]. Th1 and Th2-cells play a crucial role in directing myeloid-cell polarization [24]. CD4+ CD25+Foxp3+Treg cells physiologically curb inflammatory responses to prevent extensive self-tissue damage. Gliomas accumulate Treg-cells in their environment and utilize their immunosuppressive functions like the secretion of IL-10 and TGF-Beta for immune evasion. In addition to that Tregs suppress antigen-presenting cells and influence them to secrete TGF-Beta, thereby further inducing an immunosuppressive surrounding. [25]

Among the myeloid cell linage-derived cell populations such as myeloid derived suppressor cells (MDSCs) and dendritic cells (DCs), glioma associated microglia/macrophages (GAMS) represent the dominant cell entity. The can account for up to 45% of the tumor-mass. [26-28] This opens the possibility to render those cells a versatile target in tumor therapy.

3.1.2 Microglia

3.1.2.1 Historical Perspective and Cell Origin

Microglia are the resident phagocytes of the CNS. Pio Rio de Hortega who characterized them as an independent ramified resting cell entity with phagocytic and migratory properties introduced the term microglia [29]. Microglia account for 5-12% of the total cell population within the brain and are not uniformly distributed and shaped between different areas. The hippocampus, olfactory telencephalon, basal ganglia and substantia nigra are in particular densely populated. [30] Fate mapping studies show that 95% of microglia remained host-derived in sublethally irradiated C57BL/6 CD45.2+ new-borns, which were reconstituted with

hematopoietic cells, isolated from CD45.1+ congenic mice, suggesting that microglia are originated from myeloid progenitors in the yolk sac. [31] Underlying these results is the fact that microglia can persist in the absence of the transcription factor Myb, which is necessary for Macrophages/Monocytes developing from Hematopoietic Stem Cells (HSCs). [32] Deriving from c-Kit+ stem cells in the yolk sac, they migrate from there, colonizing the forming CNS around E9.5 and developing into CD45+/c-Kit-/CX3CR+ cells [33]. Above that, microglia maintenance and expansion were not found to be obtained by the recruitment of microglia progenitors from the circulation but to rely on self-renewal of CNS resident cells [34].

3.1.2.2 Microglia Activation States

Resident microglia in the healthy brain are displaying a ramified morphology by having a small soma and delicate cellular processes. For a long time, they have been referred to as resting since they seemed to be in a dormant state. [35] Two-Photon imaging though revealed, that in fact the term never-resting would be more accurate since microglia in the healthy brain are in a permanent surveillance state. While their somata remain fixed, their processes are highly motile and constantly in formation and withdrawal, also budding filopodia like-processes. The extracellular space is thereby scanned randomly and in a high turnover speed so that the brain parenchyma is perpetually surveyed once every few hours. Microglia even change territories by overlapping scanning of border zones, avoiding direct cell-to-cell contact within their population. [36]

There are two main purposes in microglial function, namely CNS maintenance and immune defense. Microglia function as sentinels over CNS integrity. Either the appearance of factors, which are usually absent within the CNS or the cease of dampening signals can result in triggering microglia to adapt an active phenotype. This activation is considered a change in functional phenotype rather than an actual awakening with microglia taking an amoeboid form. [37] Depending on the conditions by which microglia/macrophage activation is triggered, the resulting polarization of the cell and therefore its effector function can be diverse. Under experimental conditions, the so-called M1 or classical activation is initiated by Interferon γ (IFN-γ) in presence- or absence of bacterial products like lipopolysaccharide (LPS) and characterized by production of IL-12, IL-23 as well as high production of nitric oxide (NO) and reactive oxygen intermediates (ROI). The M2 polarization state comprises three differing states of microglia/macrophage activation being involved in tissue remodeling and immune regulation. In general, M2 cells with the exception of M2b are designated by the production of low levels of proinflammatory cytokines as IL-1, TNF-α and IL-6 as wells as low levels of IL-12. Therefore, they produce high levels of IL-10. M2a polarization is the result of an activation by IL-4 and IL-13 and is versatile in promoting a Th2-response, type II inflammation, killing

of parasites and maintaining allergy. The M2b phenotype on the other hand is induced by the exposure to a combination of immune-complexes with either TLR-agonists or IL-1R-agonsists. M2b cells simultaneously produce high levels of IL-1, TNF-α, IL-6 and IL-10, thus creating an environment in favor of inducing type two responses and exerting immunoregulatory functions. M2c polarization resulting from exposure to Il-10 drives immunosuppression, matrix deposition and tissue remodeling. [38] It has to be taken into account that the M1, M2a, M2b and M2c phenotypes seem to be extremes of a continuum, generated under experimental conditions.[38] In vivo microglia/macrophage polarization seems to underly an extreme plasticity.

This can be further underlined by the fact that already under homeostatic conditions microglia exist in a variety of morphological states between and even within different brain regions. They also display distinct inflammatory profiles, probably underlying the influence of differential microenvironmental properties. [39]

3.1.2.3 Glioma Associated Microglia and Macrophages

Whereas microglia under physiological conditions represent a distinct cell population by the separation from peripheral monocytes by the BBB, the latter gets disrupted in the course of glioma formation. Therefore, the tumor environment not solely accumulates microglia but also monocytes originated from HSCs.

In glioma models microglia/macrophages, mostly referred to as GAMs, can be distinguished by the extent of CD45 staining. While microglia were identified as CD45/low, CD11b/high cells, macrophages were characterized by a CD45/high, CD11b/high staining. [40, 41]

As previously stated, GAMs are the major cell population within the glioma microenvironment. Their number increases with malignancy-grade, being lowest in WHO Grade I tumors and highest in glioblastoma multiforme [42]. Microglia and macrophages in the context of glioma therefore do not seem to display anti-tumoral functions. On the contrary, many studies have proven that there is extensive crosstalk between GAMs and glioma cells and that both parties exhibit mutual influence. Some of those mechanisms will briefly be reviewed below.

Gliomas attract microglia to the tumor-site by the secretion of glial-derived neurotrophic factor (GDNF), a trophic factor for neuron populations within the CNS. GDNF was shown to be a potent chemoattractant for microglia. Furthermore, silencing *Gdnf* by sh-RNA in a GL261-glioma model significantly reduced the tumor size of tumor-bearing mice. [43] Monocyte chemotactic protein 3 (MCP-3), a chemokine known to promote chemotaxis of immune cells is abundant in glioma. A study conducted on human glioma-slices also correlated MCP-3 expression levels with the number of infiltrating GAMs. Hence, there was no correlation between the number of GAMs and prognosis [44]. Another well-known factor directing GAMs toward the tumor bulk is stromal-derived-factor-1 (SDF-1) [45].

Once accumulated within and around the tumor, GAMs are rendered to support glioma progression. Matrix-metalloproteases (MMPs) degrade extra-cellular-matrix, which gliomas utilize to promote brain-parenchyma invasion. Since MMPs are secreted in their inactive pro-forms, they have to be cleaved by membrane-bound metalloproteases to get activated. Gliomas stimulate microglial Toll-like-receptor 2 (TLR)-signaling via the expression of versican, an endogenous TLR-2 ligand, and thereby trigger the expression of membrane-type-1-matrix-metalloprotease (MT1-MMP), which in turn activates glioma-derived pro-MMP2. [46-48] Above that, GAMs also were shown to be the major source of MMP9 [49]. The upregulation Na(+)/H(+) exchanger isoform 1 (NHE1), a protein maintaining alkaline intracellular pH, is induced in GAMs and enhances microglia-dependent glioma infiltration. A selective knockdown of NHE1 attenuated microglia activation as well as glioma migration and proliferation [50]. Glioma cells further secrete macrophage chemoattractant protein-2 (CCL2), which acts on its receptor CCR2, found on microglia. Upon that stimulus microglia express IL-6, which in turn enhances the invasiveness of gliomas [51]. IL-10 also has its major source in GAMs and is correlated with worse prognosis [52]. Macrophage-colony-stimulating-factor (MCSF) also released by gliomas alters microglial secretome by inducing insulin-like-growth-factor-binding-protein-1 (IGFBP1). IGFBP1 then mediates angiogenesis [53]. A selective depletion of microglia has resulted in a 50% decrease of tumor-vessel density. Therefore proving, that microglia are a major angiogenetic source. [54] Within the tumor, microglia also interact with GSC. Periostin, a protein dominantly expressed by GSC recruits GAMs via integrin αvβ3 [4].

Recent findings in two different mouse-glioma models reveal that GAMs display a distinctive gene expression profile compared to the classical M1 M2a, M2b and M2c phenotypes. For that purpose, the Cd11b+ cell fraction was magnetically separated from the glioma tissue and gene expression patterns were compared to published classical gene sets. As a result, more than 1000 genes were 2-fold up-or downregulated in GAMs, showing only a partial overlap in regard to M1 or M2 patterns. One gene was shown to be amongst the highest upregulated gene in the set of GAMs, namely *Spp1*, encoding for osteopontin. Those data were affirmed by q-PCR, identifying GAMs as the main source of osteopontin transcripts both in mouse and in human [55].

3.1.3 Osteopontin

3.1.3.1 General Characteristics

Osteopontin is an extracellular matrix protein also known as Early T-lymphocyte activator (ETA-1) and Bone-Sialo-Protein-1 (BSP-1) was first discovered in 1979. [56]. The term “Osteo” means bone, while “Pontin” is referring to the Latin word for bridge, namely pons.

This depicts the capacity of osetopontin to form a bridge between cells and hydroxyapatite. [57] The term ETA-1 is originated from the observation that a 45kDa protein, now identified as a cleavage fragment of osetopontin that is secreted by activated T-helper cells is able to stimulate immunoglobulin-production in B-lymphocytes. [58] The different naming is due to the fact that the same protein was simultaneously but independently investigated within different fields, and only later it was recognized to be the same entity. In the end though, osteopontin became the most established term.

Osteopontin (OPN) is a glycosylated phosphoprotein and a member of the SIBLING (Small Integrin-Binding Ligand N-linked Glycoproteins) family. Those proteins are known to be encoded by a gene cluster on chromosomes 4 in human and chromosome 5 in mice. Osteopontin is constituted of 300 amino-acids and its size ranges from 44kDa to 66kDa depending on its pre- and post-translational modification. [59] The *Spp1* transcript is subjected to alternative splicing. This results in three secreted splice variants; OPNa, OPNb and OPNc. While OPNa represents the full transcript, OPNb is lacking exon 5 and OPNc is deprived of exon 4. The splice variants are known to exert diverse functions in healthy tissues as well as under pathological conditions. [60] The protein is further post-translationally modified by phosphorylation, glycosylation and sulphation, as well as by metalloproteinases and thrombin cleavage. Osteopontin can be detected within a number of tissues and body fluids, like bone, dentin, cartilage, uterus, vascular tissue, smooth muscle, pancreas, liver, spleen, lung, brain, milk, saliva and serum. [57, 58, 61-66]

3.1.3.2 Functions and Implications

Osteopontin is to a vast extent controlling biomineralization by binding to apatite crystal faces and inhibiting mineralization. Therefore, bones of OPN-/- mice are hypermineralized and less flexible. Osteopontin can be induced by a broad variety of cytokines and growth factors as well as environmental changes like LPS, NO, Ang II, IL-1β, TNF-α, INF-γ, TGF-β and high glucose levels, while PPARγ inhibits the osteopontin promotor [67]. Osteopontin influences innate as well as adaptive immunity.

This is for a major part due to its role in cell adhesion and leukocyte migration. α- as well as β1-integrins can be found on various cell types and bind to the integrin binding domain on osteopontin. This can result in migration as well as intracellular signaling. Above that osteopontin also acts as an extracellular ligand for CD44, which is known to promote motility and chemotaxis of immune cells [57].

Osteopontin enhances microglia proliferation and phagocytosis, while it attenuates superoxide production [68]. IL-12 release by macrophages and dendritic cells is also stimulated by osteopontin. This promotes the development of Th1-cells [69]. Also osteopontin upregulates

co-stimulatory molecules on dendritic cells, augments their HLA-DR molecule expression as well as adhesion molecules and their allostimulatory capacity [70]. OPN-/- mice fail to establish an adequate cellular immune response when challenged with intracellular pathogens like Mycobacterium tuberculosis and Lysteria monocytogenes [69]. Above that, osteopontin was found to activate the transcription factors NF-κB and AP-1, thereby directly influencing inflammatory response. Additionally, osteopontin is able to upregulate MMP-2 and MMP-9. [67]

3.1.3.3 Osteopontin in Glioma

Glioma-cell derived osteopontin exerts many functions regarding tumor expansion.

The expression levels of osteopontin within glioma cells directly correlate with cell invasiveness and tumor growth. Stably-transformed U87MG glioma cells were transformed with specific small hairpin RNA to knock down osteopontin expression, showed a diminished tumor expansion. [71] Glioma derived osteopontin is also associated with the extent of neutrophil and macrophage infiltration within the tumor [72]. Regarding its different splice variants in the context of glioma, OPNb seemed to be partially able to compensate for the absence of OPNa and OPNc. While the abrogation of the latter two reduced clonogenic survival, migration as well as proliferation and apoptosis remained unaffected. Only the knockdown of all splice variants leads to a reduction in proliferation and migration and to an increased rate of apoptosis in glioma cells [73]. Recently it has been shown that non-transformed fibroblast conditioned media transfected with *Spp1* increased microglia phagocytosis as well as active signal transducer and activator of transcription 1 (STAT1), STAT3 and STAT5. Above that, it leads to an increase in gene expression of *Arg1*, *Smad7*, *Mmp-14*, as well as *iNos* and *Irf7*. Further, it was shown that the integrin binding RGD-site of osteopontin is crucial to induce these changes in microglial activation. While osteopontin derived from non-transformed cells primarily leads to a proinflammatory microglial phenotype, glioma cells use MMP and thrombin cleavage to create a short N-terminal osteopontin fragment that induces M2-reprogramming in microglia. A knockdown of osteopontin in a C6 rat model reduced intracranial glioma growth and prevented amoeboid transformation of myeloid cells. It also reduced M2 reprogramming of GAMs [74].

Osteopontin was discovered to enhance stem cell-like phenotypes by acting as ligand upon CD44 in glioma, thereby enhancing HIF-2α activity and further inducing a hypoxia related gene signature, ultimately leading to a more aggressive glioma growth and radiation resistance [75]. Silencing of osteopontin within GSCs themselves abrogates their tumorigenic capacity in a U87-MG-mouse model by decreasing key stemness factors like Sox2 Oct-3/4 and Nanog, as well as their ability to form spheres [76].

3.1.4 Aim of the Dissertation

While there is a lot of data investigating osteopontin produced by glioma cells themselves, there is no data so far whether osteopontin originated by GAMs renders tumor growth or the tumor microenvironment, including GAMs themselves. Nevertheless, there is data on Microglia/Macrophage derived osteopontin investigating other CNS pathologies like Multiple Sclerosis, Parkinson's disease, Epilepsy, Brain-Ischemia, and Trauma, reflecting that osteopontin level is changed whenever there occurs a long-time challenge to the immune system [69, 77-81]. Research proved that immune cells like T-cells, NK-cells and dendritic cells are also capable of producing another intracellular form of osteopontin (iOPN), which differs from the secreted full length osteopontin (sOPN) in lacking the amino-terminal secretion sequence. This form is known to be differentially displayed under varying conditions, affecting the course of relaying diseases. [82-86] Up to now there has been no data to what extent GAMs produce the intracellular or the secreted form of osteopontin and how this might exert influence on glioma. Therefore, we were investigating the absence of endogenous osteopontin on glioma growth. Furthermore, we looked into the influence of host-derived osteopontin on GAM phenotype and the immune-cell population within glioma. Another aim of this study was to investigate whether GAMs do preferentially express intra-or extracellular osteopontin.

3.2 Methods

3.2.1.1 Animals

Mice were housed in the animal house facilities of the Max Delbrueck Center and handled according to governmental (LaGeSo G 0268/10, G0343/10) and MDC regulations. The mice were housed within a 12-h/12-h dark/light cycle and received food and water ad libitum. We used C57BL/6 WT mice (Charles River Laboratories) and *Spp1* knockout (OPN -/- mice) on a C57BL/6 background (The Jackson Laboratory). This Knock-out mouse strain was created by using a targeting vector containing neomycin resistance as well as a herpes simplex virus thymidine kinase gene to disrupt exons 4 through 7 of the targeted gene. The resulting construct was electroporated into 129S6/SvEvTac derived TL-1 embryonic stem (ES) cells and consecutively injected into C57BL/6 blastocysts. A crossbreeding of the created chimeric animals resulted in a C57BL/6 background. According to The Jackson Laboratory, homozygotes are known to exhibit disorganized ultrastructural wound matrix remodeling as well as defective macrophage infiltration and accumulation at sites of injury and infection. Above that, the macrophage response to mycobacteria infection, pulmonary granulomas and inflammation are impaired.

3.2.1.2 GL261 Tumor Model

The GL261 tumor model was originally induced by implanting 3-methylcholantrene pellets, a chemical carcinogen, in the brain of the C57BL/6 mice. Serial syngeneic transplantation of

small tumor pieces was performed to maintain the model [87, 88]. Tumor evolution in implanted mice is fast with an overall survival rate of under 4 weeks.

3.2.1.3 Cell Culture

Primary neonatal microglia from C57BL/6 or OPN -/- mice were prepared as described previously [89]. In brief, the cortices of new-born mice (P0-P3) were placed in HBSS and microscopically freed of meninges and blood vessels. After three consecutive washing steps, the tissue was trypsinized for 2 minutes and then the reaction was stopped by the addition of FCS containing culture medium. In the following step, the tissue was carefully dissociated with a fire-polished pipette and washed twice. The resulting glial cell mixture was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, in 75 cm2 flasks. The medium was changed every third day to remove cell debris and replenish nourishment. After nine to 12 days, when the cells were confluent, separation of the microglia from the underlying astrocytic monolayer was achieved by incubating them three days in complete DMEM containing 30% L929 conditioned medium and subsequent gentle agitation of the flask at 37 °C in a shaker-incubator at a speed of 100 rpm. Microglial cells in the supernatant were then washed and seeded in 6-well plates at a density of $1x10^6$ cells/ well. The purity of the cultures was frequently over 95%.

Glioma cells derived from the murine glioma cell line GL261 WT (National Cancer Institute) and primary microglia were cultured in Dulbecco`s modified Eagle`s medium supplemented with 10% fetal calf serum (FCS), 200mM glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin (all supplements from Invitrogen). Incubating conditions for all cells were 37°C and 5% CO2. Microglia used to assess migration were cultured without antibiotics.

3.2.1.4 Preparation of Glioma Conditioned Medium

For glioma conditioned medium GL261 cells were seeded at a density of $1x10^6$ cells in 75 cm2 tissue culture flasks and grown to 80% confluence. Then cells were replenished with fresh medium to be left on the GL261 cells for 16–18 h. The conditioned medium was aspirated and centrifuged to remove cell debris. Subsequently it was filtered using a 0.2-µm filter.

3.2.1.5 Cell Stimulation Conditions

Primary neonatal Microglia were not treated for 24 hours after seeding. Then they were treated with GCM for 24 hours.

3.2.1.6 Tumor-Inoculation

Mice used for these experiments were 11- to 15- weeks-old wildtype or OPN -/-mice. Injections were performed under anaesthesia through intraperitoneal injection of xylazine 0.02 mg/g (Rompun, Bayer) and ketamine 0.1 mg/g, (Ketanest, Pfizer). A total of 2x104 in a volume of one microliter was delivered to either one side of a brain for tumor size measurement or to both hemispheres of a brain for the rest of the experiments. C57BL/6 and OPN -/- mice were injected

in equal distribution per day and were age and sex matched. Monitoring of the mice occurred daily, scaling and behavioral assessing reflecting the mice's health condition started on day 15 past surgery. The data were integrated into a score from one, indicating an animal in normal health condition, to five, set as an absolute termination criterion in representing a mouse, which is nonresponsive to acoustic or tactile stimuli or has lost more than 20% of its starting weight.

3.2.1.7 Cell Isolation

GL261-implanted mice were sacrificed when they exceeded a score of 4 to 5, indicating a severe impairment by the tumor mass. C57BL/6 and OPN-/- mice were always euthanized in pairs, guaranteeing equality in intervals ranging from tumor-inoculations to end points. Intraperitoneal injection of 200µl pentobarbital-sodium (Narcoren, Merial GmbH) was followed by perfusion with a 0,9% NaCl solution when cells were administered to subsequent sorting or 4% paraformaldehyde when brains were used for slices. Brains were cut in ice-cold PBS (Gibco-Invitrogen), dissecting the cortex from cerebellum and olfactory bulb (Gibco-Invitrogen). The whole cortex was used for dissociation in mice without tumors, while in tumor-bearing mice only the visible tumor area was used.

First, the tissue was minced with a scalpel in ice-cold dissection buffer. The cell suspension was then passed through a 70µm cell strainer. A 22% Percoll-gradient was used to remove Myelin. ACK-Buffer was added to the remaining cells in order to lyse erythrocytes for seven minutes on ice. Cells were then washed in MACS-Buffer (PBS supplemented with 0.5% bovine serum albumin and 2 mM EDTA) and afterwards incubated with Cd11b microbeads (Miltenyi Biotec, 130-093-634) for ten minutes. MACS sorting was performed as according to the manufacturer's instructions (Miltenyi Biotec). The Flow-through was kept to assess glioma cell properties.

Spleens were processed in the same way, directly starting with the cell strainer and excluding the Percoll gradient.

Human tumor tissue resected in surgery was placed in culture medium for transportation immediately. Dissociation was achieved by using the Neural Tissue Dissociation Kit (Miltenyi Biotec) as specified by the manufacturer. For myelin-removal Myelin Removal Beads II (Miltenyi Biotec, 130-096-731) were used as instructed. Erythrocyte lysis and Cd11b sorting were accomplished as described above.

3.2.1.8 RNA Isolation and Real-Time qPCR

Total RNA was isolated using the RNeasy Mini Kit (Quiagen). Nanodrop 1000 (Thermo Scientific) was used to determine quality and yield. Complementary DNA was synthesized, using the Superscript II (Invitrogen) reverse transcriptase according to the manufacturer's instructions. The SYBR Select Mastermix (Applied Biosystems) was used to perform

quantitative RT-PCR according to the manufacturer's instructions on a 7500 Fast Real-Time PCR System (Applied Biosystems). Gene amplification was done in duplicates.

3.2.1.9 Protein Isolation and Westernblot

Whole-cell protein extracts were prepared from cultured neonatal microglia or from MACS sorted Cd11b positive cells derived from either human or murine tumor-tissue, by incubating cells on ice in RIPA buffer (Sigma-Aldrich) containing protease inhibitor cocktail tablets (Roche Diagnostics). BCA Protein Assay KIT (Pierce) was used to determine protein concentrations. 20µl of total protein per sample were mixed with Laemmli Buffer containing β-mercaptoethanol and boiled for 5 min at 95 °C. Then they were separated by 10% SDS-PAGE and afterwards wet-transferred to a PVDF membrane (Amersham). The membranes were blocked in 5% non-fat-milk in 0,05% TBS-Tween 20 for one hour and subsequently incubated with primary antibodies goat-anti-mouse-OPN (R&D Systems 1:1000) or goat-anti-human OPN (R&D Systems 1:500) over night at 4°C. Further the membrane was incubated with anti-goat horseradish peroxidase-conjugated antibody (Cell Signaling Technology, 1:2000) for two hours. Finally, the membranes were developed with the Pierce ECL Westernblot Substrate (Thermo Fisher Scientific) and the signal was detected by a Molecular Imager Gel Doc XR system (Bio-Rad Laboratories). For a loading control, membranes were stained with anti-B-Actin (Abcam, 1:10 000). Protein expression levels were analyzed using ImageJ software (National Institutes of Health).

3.2.1.10 Flow-Cytometry

Cells were prepared as described above. The data were analyzed using FlowJo10.2

3.2.1.11 Immunofluorescence Detection of Osteopontin

Immunofluorescence staining in murine specimens were exerted on 40 µm-thick free-floating brain sections. The slices were washed in PBS pH 7.4 before blocking them in PBS supplemented with 5% DKS 0, 1% TritonX for 1 hour at room temperature. Subsequently they were stained with primary antibodies for goat-anti-OPN (R&D Systems 1:100), rabbit-anti Iba1 (Wako Laboratory Chemicals, 1:125) overnight. Cy3-coupled anti-rabbit and Cy-5 coupled anti-goat antibodies (1:200) were then added to the slices for 1 hour at room temperature, followed by a staining with Hoechst (1:5000) for staining of nuclei.

3.2.1.12 LAMP staining CD31/PDF

The percentage of Ki67+ cells was normalized to the Hoechst+ cell population in 5-6 randomly distributed fields of view per tumor. The CD31+ were also counted in 5-6 randomly distributed fields of view per tumor. Vessel diameter were measured using ImageJ.

3.2.1.13 TUNEL Assay

During programmed cell death DNA gets fragmented. The TUNEL assay is based on the incorporation of modified dUTPs by the enzyme terminal deoxynucleotidyl transferase (TdT)

at the 3'-OH ends of fragmented DNA. Tumor slices were stained according to the manufacturer's instructions. Z-stacks were acquired using a Zeiss Axio Imager Z2 upright microscope. The obtained images were analyzed by quantifying the TUNEL-positive cells and normalizing them to the total tumor area.

3.2.1.14 Tumor Area Quantification

C57BL/6 and OPN-/- tumor-bearing mice (n=8 per group), which were inoculated simultaneously with GL261 cells were sacrificed on the same day whenever one mouse reached a score of five as described above. Brains were fixed after perfusion and cut into 40 μm-thick slices. Slices were immunofluorescence stained with Hoechst (1:5000). Tumor area was then measured as percentage of tumor-infiltrated brain area per slice.

3.2.1.15 Analysis of Survival

For investigating potential effects of a total OPN-/- in the tumor-microenvironment on invasion and tumor-growth, C57BL/6 and OPN-/- mice (n=11 per group) inoculated at equal time points were also sacrificed the same day. Instead of measuring the time between inoculation and death for reasons of animal welfare, an objective score consisting of parameters as for example loss of shiny fur, hydrocephalus or weight decline was used. Whenever a mouse reached a score of five this day was assessed as endpoint.

3.2.1.16 Image Acquisition and Processing

Images of immunofluorescence stainings of brain slices from GL261-injected C57BL/6 mice were taken using a Leica confocal microscope. Pictures were taken in a 40x magnification with at least ten random fields of interest (tumor area) in two slices per brain (n=5). ImageJ software was used to count Iba1 and osteopontin positive labelled cells, as well as double-positive labelled cells.

3.2.1.17 Statistical Analyses

The statistical tests were carried out with GraphPad Prism 7. The significance level is set at five percent.

3.2.2 Resultsf

3.2.2.1 Microglia Express a Secreted and an Intracellular Form of Osteopontin in Culture

To investigate microglial osteopontin protein production under basic cultural and stimulation conditions, cultured neonatal Microglia were treated with GCM for the period of 24 hours. Westernblot analysis revealed a double band, implying the existence of a secreted as well as an intracellular form of osteopontin, represented by the slightly larger 60kDa (sOPN) and the 56kDa (iOPN) in cultured neonatal microglia (Fig.2C). For all figure references, the reader is advised to refer to the paper in the attachment. Those bands were not changed under the influence of GCM and absent in OPN-/- microglia and GL261 cells.

3.2.2.2 In Glioma Associated Microglia/Macrophages the Secreted Form of Osteopontin is Predominant

There were very low amounts of intracellular and secreted osteopontin detectable in the Cd11b+ cells from naive brains by Westernblot. In contrary to this, examining the Cd11b+ cell fraction isolated from GL261 tumors by preceding MACS, revealed that solely the secreted form of osteopontin was detectable on the Westernblot membrane, whereas the intracellular form was completely absent (Fig.2C). Comparing the sOPN protein levels in Glioma associated Microglia/Macrophages to those of the residual Cd11b+-depleted tumor cell mass, there was no significant difference in the levels of sOPN between these groups.

3.2.2.3 Osteopontin is Predominantly Expressed in GAMs Located Within the Tumor Core.

We used an ostepontin/Iba1 co-staining to localize osteopontin in sections of GL261-tumor-bearing mice brains. Our analysis shows that osteopontin is mainly located in the tumor core but also to a high abundance in the tumor border. Focusing on the tumor core, 62% of total osteopontin is found inside of GAMs and 33% of all Cd11b+ are positive for osteopontin (Fig.1F). There was also osteopontin expression in PDGFRβ+ vessel-lining pericytes found to a lower degree (Fig1G). The possibility that osteopontin was primarily phagocytosed was excluded by using LAMP-1 co-staining.

3.2.2.4 Osteopontin is Expressed by Brain Resident Microglia and Periphery-Derived Monocytes/Macrophages Within the Tumor

We used a $Cx3cr^{GFPwt}/Ccr2^{RFP/wt}$ mouse model to distinguish between brain resident microglia and periphery-derived macrophages/monocyes in a GL261 tumor model. *Spp1* was expressed highly in GFP+/RFP- microglia as well as in GFP low/RFP+monocytes/macrophages within the tumor core. (Fig.2B)

3.2.2.5 Depriving the Tumor Microenvironment of Osteopontin Enhances Tumor Growth and Disease Progression

Comparing the disease progression in GL261 tumor-inoculated OPN-/-mice to those in wild type mice, an objective score as described above was used to assess health conditions in mice. The data reveals a significant difference in the OPN-/- strain compared to the wild type strain by displaying a more rapid decline in the first (Fig.3A). Differences in tumor size were measured in 40-µm-thick-sections from tumor-bearing mice. Then the percentage of brain area infiltrated by the tumor was measured. Consistent with the findings in disease evolution, tumor area in OPN-/- mice was also significantly more extended than in wild type mice (Fig.3C).

3.2.2.6 The Lack of Osteopontin Does Not Increase Proliferation but Decreases Tumor Cell Apoptosis

We used a Ki67-staining to measure the percentage of proliferating cells in OPN-/- and wild type tumor-bearing-mice. There was no significant difference between the mouse strains. Therefore, there was a decrease of TUNEL+ cells in OPN-/- animals, revealing a decreased rate

of apoptotic tumor cells (Fig.3H).

3.2.2.7 Cytokine Levels in GAMs Do Not Differ Between OPN -/- Mice and Wild type Mice

Attempting to find an explanation for the divergence in disease progression and tumor size between those two mouse strains, analysis of cytokine-levels known to be rendered in GAMs in the context of glioma were measured by q-PCR. There was no significant difference in cytokine levels between those mouse strains (Fig.6).

3.2.2.8 The Absence of Host-Derived Osteopontin Increases Microglia Density in Glioma

We analyzed the composition of cells forming the tumor-microenvironment in the presence and absence of OPN using flow-cytometry on either tumor-bearing wild type or OPN-/- mice. While there was no difference in the T-cell population, there was an increase in Cd11b+/Cd45low brain-resident microglia in OPN -/- compared to wild type mice (Fig.5B).

3.2.2.9 Osteopontin Influences the Tumor Microvasculature

In OPN -/- tumor-bearing mice, we discovered a lower blood-vessel density with a trend toward a larger vessel size. Our findings also revealed a lower number of PDFGRβ+ pericytes in OPN -/- mice (Fig.4).

3.3 Discussion

Initially microglia were thought to play an important part in tumor defense, being the dominating immune cells in the CNS. However, microglia have been acknowledged as versatile factors in glioma expansion and progression. Complex relations between glioma cells and microglia dominate and alter the tumor-microenvironment [90-92]. For this reason, the emphasis of this study lies on this mutual influence, trying to find a variable, which might allow to render GAMs to adapt a tumor impeding instead of a tumor supporting phenotype.

Osteopontin has been investigated and discussed as a possible target in glioma research over the past years, though disregarding GAMs as a main source and primarily focusing on osteopontin released by glioma cells or its function in maintaining stem cell character [71-73, 75, 76, 93-95]. We are the first group gathering insight into osteopontin produced by GAMs. As shown previously by our laboratory [55], *Spp1* is amongst the most upregulated genes in GAMs and correlates inversely with patient-survival according to the Repository for Molecular Brain Neoplasia Data (REMBRANDT). Another distinction regarding osteopontin in the context of glioma has not been made so far, namely the discrimination between the secreted and the intracellular form of osteopontin.

In this study it becomes apparent that staining for microglial osteopontin on a Westernblot membrane detects a characteristic double band resembling the one previously described by [83] in dendritic cells. This suggests the existence of an intracellular as well as a secreted form of osteopontin in cultured and freshly isolated microglia. We identified a change in the expression of sOPN and iOPN by microglia under the influence of glioma, showing for the first time that

GAMs solely express the secreted form of osteopontin. Summarizing these results, the balance of sOPN and iOPN, being present under unstimulated conditions, is shifted under the influence of glioma and leads to the prevalence of sOPN throughout the tumor core.

So far, there exists no information to what extent the enhancement or suppression of intracellular osteopontin renders microglia phenotype. Findings by [96] revealed endogenous osteopontin production maintains macrophage function and differentiation. Macrophages silenced for osteopontin showed impaired migration, increased susceptibility for apoptosis, and a dedifferentiated phenotype that could not be reversed by adding recombinant osteopontin. Similar findings were made by [84] who have created a selective KI (knock-in) mouse for iOPN, proving that iOPN deficiency in NK cells impairs maturation, function, and survival. Altogether, these results suggest intracellular osteopontin is essential for innate immune cells to mature and exert their effector functions. Regarding the fact, that GAMs seem to be impaired in their ability to express iOPN, this implies they are possibly rendered in their state of differentiation by the tumor and therefore fail to suppress tumor growth. Determination of cytokine levels by q-PCR further revealed that GAMs completely lacking both forms of osteopontin are not comprised in their functions related to cytokine production, since there was no significant difference to GAMs being capable to produce osteopontin.

Apart from that, our results regarding the tumor growth in OPN -/- mice are consistent with the hypothesis that intracellular osteopontin might exert anti-tumor functions when expressed by GAMs. Compared to wild-type mice, tumor growth and disease progression were significantly faster in OPN -/- mice. While the upregulation of osteopontin in glioma cells themselves enhances tumor growth [76, 93], a complete abrogation of osteopontin in the tumor microenvironment leads to the opposite effect and could possibly underlie the absence of intracellular osteopontin.

Further looking into host-derived vs tumor-derived osteopontin functions, findings in tumors other than glioma, also show that abolishing osteopontin production in the host-organism increases tumor growth. [97-99] Injecting cutaneous squamous cell carcinoma cells into wild-type and OPN-/- mice showed that squamous cell carcinoma developed more frequently in OPN-/- mice than in wild type mice. Similar to our findings, tumors in OPN -/- mice were significantly larger than in the control-group [97]. In a model of hepatocarcinogenesis, mice were injected intraperitoneally with Diethylnitrosamine (DEN). Again, there was a significant increase in tumor size and tumor number in OPN-/- mice compared to wild type mice. Kaplan-Meier survival curves showed a decreased survival time of OPN-/- mice. [97] In addition, it has recently been suggested that upregulation of iOPN might enhance anti-tumor response in immune cells [84, 98].

Conclusively, finding a possibility to selectively promote the expression of iOPN in the tumor microenvironment could provide a chance to target glioma.

There are several limitations to our study that leave space for further investigations. Up until now it is not known to what extent the concept of iOPN and sOPN can be translated into the human setting. Although human iOPN has been detected by confocal microscopy there is no knowledge where human iOPN appears on a SDS page and whether the lack of osteopontin in a human host has the same consequence as in mice.[85] Furthermore, compared to human disease evolution GL261 tumors do not mimic human tumor heterogeneity. As implied in other tumor entities, osteopontin might exert different function at different stages of tumor evolution [97]. This has not been considered in this project. What should be stressed again is the fact that a high *Spp1* expression does not reflect a glioma specific microglia phenotype. As mentioned previously *Spp1* is upregulated whenever there is a long time challenge to the immune system [69, 77-81]. Thus, studying the function of osteopontin in glioma might skip a step to the basal understanding of the protein in immune response and therefore in immune cell maturation. The next step would be to study the influence of iOPN and sOPN on basal microglia functions. Another interesting question would be to investigate if an upregulation of iOPN in glioma could render microglia into exerting anti-tumor functions. Then finding a way to selectively promote iOPN in glioma would be an immunotherapeutic approach. Translating this research into a human setting will be challenging due to the limited existing knowledge regarding the existence and function of iOPN and sOPN in human.

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4 Declaration of Own Contribution

4.1 Declaration of own Contribution to the Top-Journal Publication

Nina Schwendinger contributed the following to the below listed publications:

The development of the idea to the project was created in cooperation with Mr. Frank Szulzewsky. Moreover, there occurred a joint planning of the experiments. I did the testing for diverse antibodies for Westernblot and Immunochemistry as well as for FACS. In addition, I independently performed the Westernblot experiments. The immunohistochemical stainings for OPN and Iba1 and their analysis were partly done by me and partly done by Mr. Szulzewsky. Apart from that I was responsible for the quantification of tumor area in the mouse models, isolation of Microglia/Macrophages via MACS and preparation and staining of Microglia for FACS-analysis. The development of the mouse-score-system, monitoring of disease symptoms and producing GL261-conditioned medium was independently done by me. The design and testing as well as performing and analysis of qRT-PCRs was partly done by me or the other first author. The statistical analysis of the in vitro and in vivo generated data was common work of Mr. Szulzewsky and me. The interpretation of the results and the draft of the manuscript were done in cooperation with the other first-author and the co-authors. The stainings for Ki67, CD31, PDGFRbeta, GFAP, AQP4, GRASP65 and LAMP1 as well as the TUNEL-Assay were solely done and interpreted by Frank Szulzewsky. The stainings in human samples are also the work of Frank Szulzewsky. FACS analysis FlowJo was done by Dilansu Günekaya.

Publication : Frank Szulzewsky, Nina Schwendinger, Dilansu Güneykaya, Patrick J Cimino, Dolores Hambardzumyan, Michael Synowitz, Eric C Holland, Helmut Kettenmann. **Loss of host-derived Osteopontin creates a glioblastoma-promoting microenvironment, Neuro-Oncology, November 2017, 19:6(252).**

Signature, date and stamp of supervising university professor / lecturer

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Signature of doctoral candidate

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5 Statutory Declaration

"I, Nina, Schwendinger, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic The Influence of Host-Derived Osteopontin on Glioma, independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I am aware of the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice and that I commit to comply with these regulations.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date Signature

6 Extract from the Journal Summary List (ISI Web of KnowledgeSM)

Journal Data Filtered By: **Selected JCR Year: 2017** Selected Editions: SCIE,SSCI
Selected Categories: **"ONCOLOGY"** Selected Category Scheme: WoS
Gesamtanzahl: 222 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	CA-A CANCER JOURNAL FOR CLINICIANS	28,839	244.585	0.066030
2	NATURE REVIEWS CANCER	50,407	42.784	0.079730
3	LANCET ONCOLOGY	44,961	36.418	0.136440
4	JOURNAL OF CLINICAL ONCOLOGY	156,474	26.303	0.285130
5	Nature Reviews Clinical Oncology	8,354	24.653	0.026110
6	Cancer Discovery	11,896	24.373	0.065350
7	CANCER CELL	35,217	22.844	0.096910
8	JAMA Oncology	5,707	20.871	0.027770
9	ANNALS OF ONCOLOGY	38,738	13.926	0.095780
10	JNCI-Journal of the National Cancer Institute	37,933	11.238	0.052550
11	Journal of Thoracic Oncology	15,010	10.336	0.033280
12	CLINICAL CANCER RESEARCH	81,859	10.199	0.132210
13	SEMINARS IN CANCER BIOLOGY	6,330	10.198	0.010740
14	LEUKEMIA	25,265	10.023	0.059580
15	NEURO-ONCOLOGY	10,930	9.384	0.030350
16	Cancer Immunology Research	4,361	9.188	0.021180
17	CANCER RESEARCH	139,291	9.130	0.130190
18	Journal for ImmunoTherapy of Cancer	1,675	8.374	0.007130
19	BIOCHIMICA ET BIOPHYSICA ACTA-REVIEWS ON CANCER	5,276	8.220	0.009300
20	Blood Cancer Journal	1,804	8.125	0.007660
21	CANCER TREATMENT REVIEWS	7,870	8.122	0.015820
22	Molecular Cancer	10,301	7.776	0.017280
23	INTERNATIONAL JOURNAL OF CANCER	51,800	7.360	0.071870
24	Journal of Hematology & Oncology	4,098	7.333	0.009750
25	EUROPEAN JOURNAL OF CANCER	29,883	7.191	0.050170
26	ONCOGENE	66,411	6.854	0.075960
27	CANCER	68,221	6.537	0.074740
28	CANCER LETTERS	29,311	6.491	0.042280
29	Journal of the National Comprehensive Cancer Network	5,143	6.471	0.017530
30	Advances in Cancer Research	2,343	6.422	0.003690
31	JOURNAL OF PATHOLOGY	16,156	6.253	0.024060

7 Copy (print) of the Paper

https://doi.org/10.1093/neuonc/nox165

8 Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten

9 List of Publications

Publikation : Frank Szulzewsky, Nina Schwendinger, Dilansu Güneykaya, Patrick J Cimino, Dolores Hambardzumyan, Michael Synowitz, Eric C Holland, Helmut Kettenmann. **Loss of host-derived Osteopontin creates a glioblastoma-promoting microenvironment, Neuro-Oncology, November 2017, 19:6(252).**

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