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BY
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AALBORG UNIVERSITY
DENMARK

UNDERSTANDING GLIOBLASTOMA STEM-LIKE CELLS IN QUEST OF A TARGET TO DIRECT TREATMENT

by

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LIST OF PUBLICATIONS

J.M. Gudbergsson, M. Duroux: Cripto-1 localizes to dynamic and shed filopodia associated with cellular migration in glioblastoma cells. *Eur. J. Cell Biol. (2019).*

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ENGLISH SUMMARY

Glioblastoma multiforme (GBM) is the most common and deadly tumor of the central nervous system with a median overall survival of only 12-15 months from time of diagnosis. Treatment consists of maximum safe resection surgery followed by radio- and chemotherapy. Despite this heavy treatment tumor recurrence remains ubiquitous with the recurrent tumors being more aggressive and resistant to treatments, underscoring the need for better understanding of GBM biology and improved treatment strategies for GBM patients. GBM tumors are highly different from each other and within each tumor a high location-dependent heterogeneity exists. On a cellular level, a tumor cell hierarchy is formed partially in resemblance to that of a normal organ, with GBM stem cells (GSC) at the apex orchestrating the tumor growth and composition. GSCs exploit pathways that are highly active during normal embryonal development, including functions such as cellular migration, asymmetric cell division and cellular differentiation.

In this thesis, the objective was to identify proteins on GSCs to direct treatments towards these hard-to-reach cells. We have here included four manuscripts that represent the fundament of our research to achieve this goal. Initially, we had identified cripto-1 as a potential target towards GSCs and investigated its subcellular localization in an overexpression model to see where this protein might be situated in order to evaluate whether it could be used as a target. We showed that cripto-1 was mainly localized to the plasma membrane and was highly present in membrane-derived filopodia structures. Next, while examining cripto-1 in wildtype (non-overexpressed) GBM cells, we saw some unexpected binding of the cripto-1 antibodies used. This led to an evaluation of four different cripto-1 antibodies, reporting different results from each of these antibodies, and thus a discontinuation of cripto-1 research was decided.

In order to better identify new and relevant targets, we needed more translatable GBM cellular models for both *in vitro* and *in vivo* studies. Here, we characterized a patient-derived tumorsphere model that showed intercellular heterogeneity both *in vitro* and *in vivo* and could be used for therapeutic assessment *in vitro*. Next, we expanded on this model and set up a larger *in vivo* study where we examined how conventional GBM treatments (radiotherapy and temozolomide) affected the cellular composition of the GBM tumors. Through multicolor flow cytometry and FlowSOM clustering analysis, we showed that an upregulation of CD44 was seen in all treatment groups compared to the untreated group. This could indicate that CD44 could become a target for future directed treatments, however, we still have a long road ahead of us. In the overall discussion presented in this thesis, I cover some contextual topics that include modeling of GBM tumors and targeting GSCs. I believe that the results presented here have contributed to the overall understanding of cripto-1 protein in GBM cells and the challenges that come with it. Our investigation of GBM cell plasticity in response to conventional treatment has paved a new way of studying such phenomenon with flow cytometry and cluster analyses which could potentially be adapted to clinical settings.

DANSK RESUME

Glioblastoma multiforme (GBM) er den mest almindelige og dødbringende tumor i centralnervesystemet med en gennemsnitlig overlevelse på kun 12-15 måneder fra diagnose. Behandlingen består af kirurgi efterfulgt af stråle- og kemoterapi. På trods af den hårde behandling sker der i alle tilfælde genvækst af tumoren, idet de tilbagevendende tumorceller er mere aggressive og resistente over for behandling, hvilket understreger behovet for bedre forståelse af GBM biologi og bedre behandlingsstrategier for GBM patienter. GBM tumorer er meget forskellige fra hinanden, og i hver tumor findes en høj lokaliserings-afhængig cellulær heterogenitet. På celleniveau dannes et tumorcellehierarki som på mange måder ligner det som ses i et normalt organ, med GBM stamceller (GSC) i toppen som orkestrerer tumorvækst og sammensætning af celler. GSC'er gør brug af signaleringsveje der oftest ses aktive under normal embryonal udvikling, hvilket inkluderer funktioner såsom cellulær migration, asymmetrisk celledeling og cellulær differentiering.

Formålet med denne afhandling var at identificere proteiner på GSC'er for at målrette behandling mod disse resistente celler. Vi har her inkluderet fire manuskripter, som danner grundlaget for vores forskning for at nå dette mål. Oprindeligt havde vi identificeret cripto-1 som et potentielt mål mod GSC'er og undersøgt dets subcellulære lokalisering i en overekspressionsmodel. Formålet var at se hvor dette protein er placeret for at evaluere, om det kunne bruges som et mål i behandling. Vi fandt at cripto-1 hovedsageligt var lokaliseret til plasmamembranen og var meget til stede i cellemembran-deriverede filopodia-strukturer. Dernæst, mens vi undersøgte cripto-1 i vildtype (ikke-overudtrykte) GBM celler, så vi uventet binding af de anvendte cripto-1-antistoffer. Dette førte til en evaluering af fire forskellige cripto-1-antistoffer der alle viste forskellige resultater, hvilket førte til en afvikling af vores cripto-1-forskning.

For bedre at kunne identificere nye og relevante mål havde vi brug for bedre cellulære GBM modeller til både *in vitro* og *in vivo* eksperimenter. Her karakteriserede vi en patient-afledt tumorsphere model, der viste intercellulær heterogenitet både *in vitro* og *in vivo,* og kunne bruges til evaluering af behandling *in vitro*. Dernæst udvidede vi på denne model og udførte et større *in vivo* eksperiment, hvor vi undersøgte, hvordan konventionelle GBM behandlinger (strålebehandling og temozolomid) påvirkede den cellulære sammensætning af GBM tumorer. Gennem avanceret flowcytometri og FlowSOM-analyse viste vi, at der blev set en opregulering af CD44 i alle behandlingsgrupper sammenlignet med den ubehandlede gruppe. Dette kan indikere, at CD44 kan blive et mål for fremtidige målrettede behandlinger. I den samlede diskussion der præsenteres i denne afhandling, dækker jeg nogle kontekstuelle emner, der inkluderer modellering af GBM tumorer og målretning mod GSC'er. Samlet set har vi med disse resultater bidraget til en udvidet forståelse af cripto-1 proteinet i GBM celler samt vist en ny måde at lave en detaljeret undersøgelse af behandlingsresistente tumorceller med flow cytometry som potentielt kan implementeres i klinikken.

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First of all, I would like to thank Meg Duroux and the Department of Health Science and Technology at Aalborg University for the opportunity to obtain a PhD within brain tumor biology. A sincere thank you to Trine Fink for kindly supervising me through the last months of my PhD and assisting with administrative tasks.

A special thanks goes to Kasper Bendix Johnsen who has been a mentor and a close colleague for several years, not to mention a great friend since we attended high school together. I would also like to thank Serhii Kostrikov for being my partner in crime during my two research stays in Copenhagen, always ready to work late hours and discuss science over a beer. Furthermore, a huge thanks goes to Frederikke Petrine Fliedner for introducing me to brain orthotopic surgical implantation of glioblastoma cells and MRI.

I would also sincerely like to thank Professor Thomas Lars Andresen (DTU) and Professor Andreas Kjær (KU) to allow me to be a part of their research teams at the Panum Institute. In that regard, I would like to thank all my close collaborators and friends at the Center for Nanomedicine and Theranostics (DTU) and Cluster for Molecular Imaging (KU) for making my stay a complete pleasure by providing helping hands and scientific inspiration. These persons include, but are not limited to: Esben Christensen, Lars Ringgaard Petersen, Anders Elias Hansen, Lotte Kellemann Kristensen, Carsten Haagen Nielsen, Michelle Westergaard Kaijer, Anne Mette Fisker Hag, and the skilled scientists at Core Facility for Integrated Microscopy (CFIM, KU). Several additional persons contributed significantly on a personal level to making my stay a pleasure, and I think you all know who you are.

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I would like to thank all my collaborators from AAU and outside AAU that have not yet been mentioned, including Gunna Christiansen (AU/AAU) and Leonid Gurevich (AAU), Kamilla Ellerman Jensen and Petra Hamerlik from the Brain Tumor Biology group at the Danish Cancer Society, and Bjarne Winther Kristensen (SDU/OUH).

Last but not least, a heartfelt thank you goes to my amazing girlfriend Margret for always supporting me through tough times; this could not have been done without her. Also, thank you to my lovely family and friends for loving support and understanding when I have been dug into a scientific hole for longer periods of time.

PREFACE

The work presented in this PhD thesis is based on research mainly carried out at Aalborg University as a part of Laboratory of Cancer Biology. The research conducted is presented in four separate manuscripts. Parts of the experiments performed in Manuscript III and all experiments in Manuscript IV were done during my two research stays in Copenhagen. The first stay was from August 2017 to December 2017, and the second was from September 2018 to December 2018. During these stays I was a part of Center for Nanomedicine and Theranostics at the Technical University of Denmark (DTU) lead by Professor Thomas L. Andresen who has a tight collaboration with Cluster for Molecular Imaging at University of Copenhagen lead by Professor Andreas Kjær. All *in vivo* experiments were performed in their animal facilities at the Panum Institute.

The thesis has been submitted as a compilation thesis (collection of articles) in concordance with the requirements set by the Doctoral School in Medicine, Biomedical Science and Technology at Aalborg University. The thesis consists of a broad introduction to the field (Chapter 1) followed by the thesis objectives (Chapter 2). Chapter 3 consists of a brief summary of the findings from each manuscript presented as extended unstructured abstracts. Chapter 4 presents a discussion of key findings from the manuscripts and a relation of these to methodological or conceptual issues. Lastly, the thesis ends with concluding remarks and future perspectives (Chapter 5).

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Manuscript II: **J.M. Gudbergsson**, M. Duroux: An evaluation of different Cripto□1 antibodies and their variable results. *J. Cell. Biochem. (2019).*

Manuscript III: **J.M. Gudbergsson**, S. Kostrikov, K.B. Johnsen, F.P. Fliedner, C.B. Stolberg, N. Humle, A.E. Hansen, B.W. Kristensen, G. Christiansen, A. Kjær, T.L. Andresen, M. Duroux: A tumorsphere model of glioblastoma multiforme with intratumoral heterogeneity for quantitative analysis of cellular migration and drug response. *Exp. Cell Res. 379 (2019) 73–82.*

Manuscript IV: **J.M. Gudbergsson**, E. Christensen, S. Kostrikov, T. Moos, M. Duroux, A. Kjær, K.B. Johnsen, T.L. Andresen: Conventional treatment for glioblastoma reveals persistent CD44+ subpopulations. [*Manuscript submitted*]

Other activities:

J.M. Gudbergsson, K. Jønsson, J.B. Simonsen, K.B. Johnsen: Systematic review of targeted extracellular vesicles for drug delivery – Considerations on methodological and biological heterogeneity**.** *J. Control. Release. 306 (2019) 108–120.*

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LIST OF ABBREVIATIONS

BMP – Bone morphogenic protein
CD – Cluster of differentiation
CNS – Central nervous system
DNA – Deoxyribonucleic acid
ECM – Extracellular matrix
EGFR – Epidermal growth factor receptor
FDA – Food & Drug Administration
GBM – Glioblastoma multiforme
GEM – Genetically engineered models
GPI - Glycosylphosphatidylinositol
GSC – Glioma stem-like cell
HIF-2α – Hypoxia-inducible factor 2α
IHC – Immunohistochemistry
IR – Irradiation
IRF3 – Interferon regulatory factor 3
JAK – Janus kinase
MGMT – O6-methylguanine DNA methyltransferase
NF-kβ – Nuclear factor kappa-light-chain-enhancer of activated B cells
PRC2 – Polycomb repressive complex 2
RNA – Ribonucleic acid
STAT3 – Signal transducer and activator of transcription 3
TGFβ – Transforming growth factor β
TMZ – Temozolomide
VEGF-A – Vascular endothelial growth factor A
SFRP1 – Secreted frizzled related protein 1

CHAPTER 1. INTRODUCTION

Glioblastoma Multiforme (GBM) is the most frequent malignant tumor of the central nervous system (CNS), representing 46.6 % of all malignant CNS tumors in the United States in the years 2009 – 2013. The population incidence in United States is 3.2 per 100.000 and median age of diagnosis is 64 years. Overall survival rates are poor with a median survival of 15 months from diagnosis. [1] GBM is classified as primary (or *de novo*) with a short clinical history or secondary which arises from lower grade astrocytomas [2]. Three transcriptomic profiles have further subdivided GBM into classical, proneural and mesenchymal subtypes [3]. Current treatment consists of maximum safe resection surgery followed by radiotherapy plus concomitant and adjuvant chemotherapy with the alkylating agent temozolomide (TMZ) [4]. In spite of such heavy treatment tumor recurrence remains inevitable, often accompanied by several satellite tumors forming at distant locations in the brain (Figure 1.1) [4,5]. Extensive research efforts and clinical trials have been conducted in order to improve current treatment outcomes, however, most have failed. A positive prognostic indicator for TMZ treatment response is the hypermethylation, and thus inactivation, of the O-6-methylguanine-DNA methyltransferase (MGMT) promoter [6]. A recent phase 3 clinical trial showed that the combination of the alkylating nitrosurea lomustine combined with TMZ might be superior to TMZ alone in GBM patients with hypermethylated MGMT promoter [7]. Another therapeutic agent that seemed promising was the anti-VEGF-A immunotherapy bevacizumab. However, bevacizumab did not show significant results and induced adverse effects in some GBM patients [8,9]. Since bevacizumab showed effects in few patients, it has still been FDA approved as a last-line treatment, highlighting the desperate need for improvement [10].

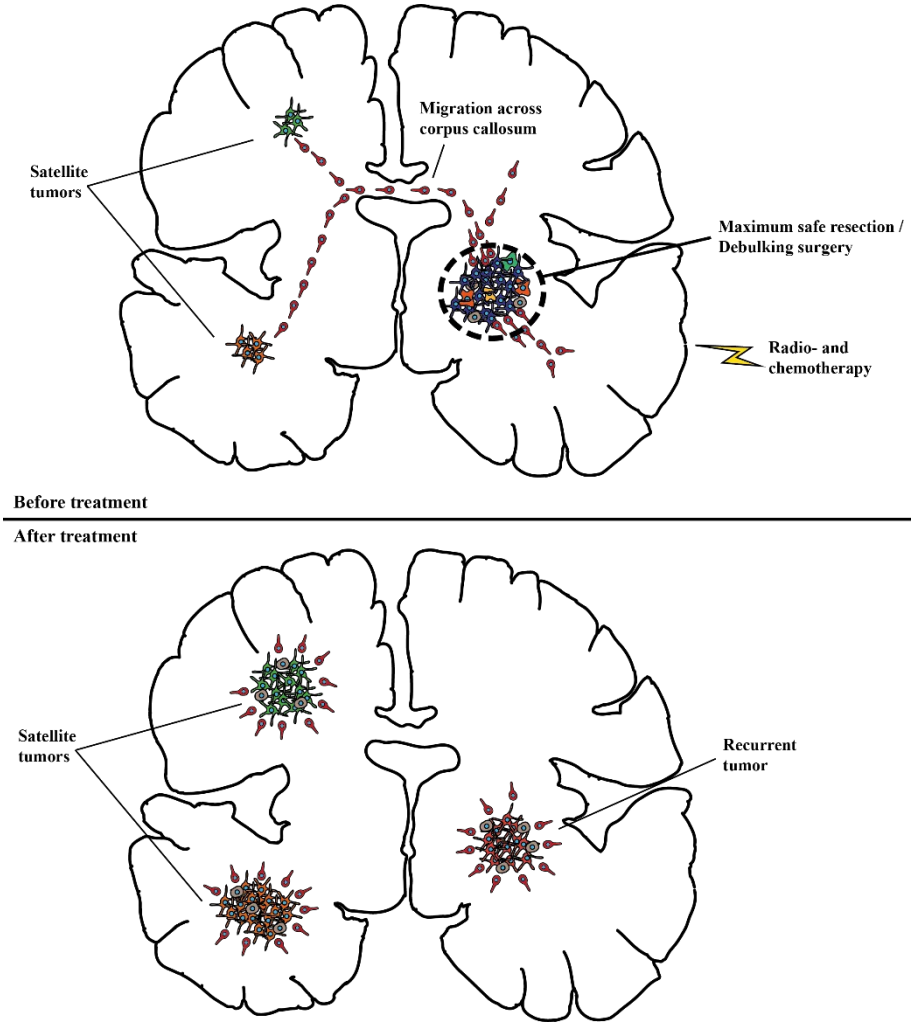

Figure 1.1. *Potential situation before and after treatments for GBM, showing migration, satellite tumor formation and recurrence of primary tumor after surgery.*

1.1. GLIOBLASTOMA STEM-LIKE CELLS: THE WHAT, WHERE AND HOW

GBM tumors are highly heterogeneous consisting of various tumor cells, healthy tumor-associated cells, different immune cells, and a small fraction of highly malignant GBM stem-like cells (GSCs). GSCs were initially discovered in the beginning of the 2000's where neural stem-like cells were found in cortical gliomas

and GBM [11,12]. Generally, GSCs are defined as highly malignant tumor cells with stem cell properties such as asymmetric cell division, unlimited replication capacity, capable of differentiation and able of tumor formation [13]. These cells have been implicated in processes such as invasion, angiogenesis, immune evasion and therapeutic resistance [13] (Figure 1.2). Several protein markers have been proposed to identify GSCs such as CD133, Nestin, Oct-4, Sox2 and CD44, most of which have previously been associated with neural stem cells and radial glial cells [14–17]. Within a single GBM tumor, several subtypes of non-stem cells and GSC subtypes have been identified adding to the immense complexity of the composition of GBM [18].

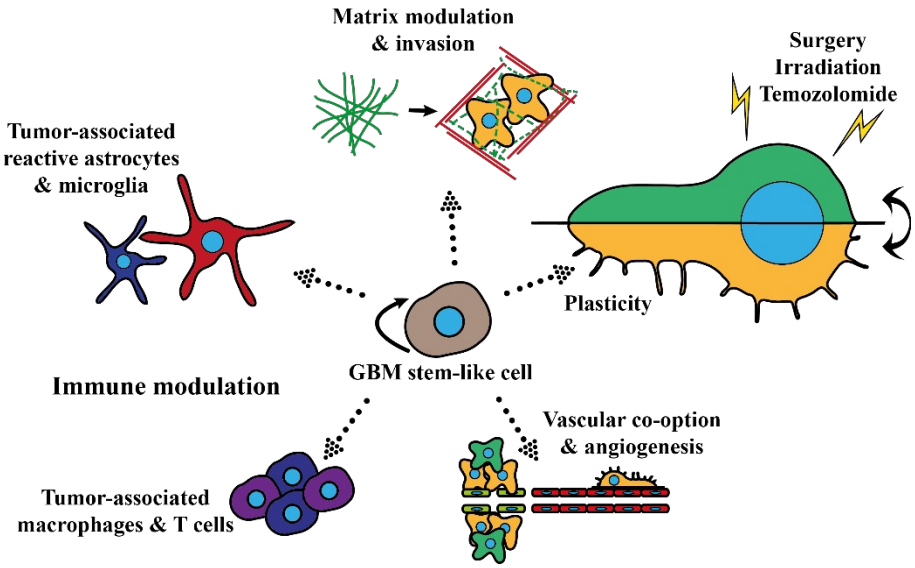

Figure 1.2. *Overview of key functions orchestrated by GSCs in glioblastoma pathogenesis and progression.*

1.1.1. STEM-LIKE CELLS AT THE APEX OF TUMOR CELL HIERARCHY

The event of tumor formation is not well elucidated, but two theories currently exist; the stochastic (or clonal evolution) theory and the hierarchical cancer stem cell theory. The stochastic theory proposes a clonal selection of tumor-initiating cells that in the end are quite similar (stemness-wise) or homogenous and can all form a tumor, following the 'survival of the fittest' principle. The cancer stem cell theory proposes that tumor formation is more like the process of organogenesis during embryonal development in which cancer stem cells are responsible for cellular differentiation, creating a highly heterogeneous tumor (Figure 1.2). Since most

tumors to date have shown intratumoral cellular heterogeneity including cancer stem cell populations, the cancer stem cell theory has over the past decade gained more support than the stochastic theory. In GBM, much evidence exists on the intercellular heterogeneity in which GSCs have been proposed to dictate the formation, composition and progression of GBM tumors [13,19,20].

1.1.2. THE GSC NICHES

The thought and fascination of the heterogenic development of gliomas is not new. In 1938, Hans Scherer compiled his work on glioma morphology from 100 patient autopsies and described tumor cells in white matter tracts and clustering around vasculature in the tumor periphery [21]. Today, we know much more of which cell types occupy the different spaces or *niches*. The GSCs have been found residing in specific niches, such as the perivascular niche, perinecrotic or hypoxic niche and the less elucidated invasive niche, as illustrated in Figure 1.3 [20]. In the perivascular niche, GSCs have been found to interact with surrounding cells to promote self-growth and cell invasion [22,23], but it has long been debated which type of blood vessel the GSCs prefer to harbor. Recently, it was suggested that GSCs cluster around arterioles due to the notion of arterioles not allowing oxygen exchange with surrounding tissue, thus creating a hypoxic state [24]. This hypoxic periarteriolar niche has been hypothesized of being the main type of perivascular GSC niche and has been rationalized due to its high similarity to hematopoietic stem cell niches in the bone marrow [24–26]. Hypoxia is a key inducer of stemness in all types of cancer, including GBM [27,28]. The perinecrotic niche is also called the hypoxic niche and has been shown to be enriched in GSCs. Maintenance of GSCs in the perinecrotic niche has been attributed to hypoxia-inducible factor 2α (HIF-2α) through activation of CD44 [29,30]. CD44-induced HIF-2α activation was also shown to induce a pseudo-hypoxic phenotype in perivascular GSCs [29]. Other members of the HIF family also regulate the GSC stemness through embryonal pathways such as Notch-Jagged and TGFβ [28,31]. A less defined enrichment site of GSCs is the invasive niche, most likely due to difficulties in studying the invasive cells in GBM patients [20]. It is widely shown that invasive cells in GBM possess stem-like characteristics, and several motility-associated pathways have been discovered as key in GSC invasion, including Ephrin, TGF-β and Integrins resulting in Rho and Ras-mediated activation of NF-kβ, JAK/STAT3, IRF3 and PRC2 [32]. Also in GBM invasion, hypoxia has been shown to induce an invasive stem-like phenotype promoting mesenchymal traits [31,33].

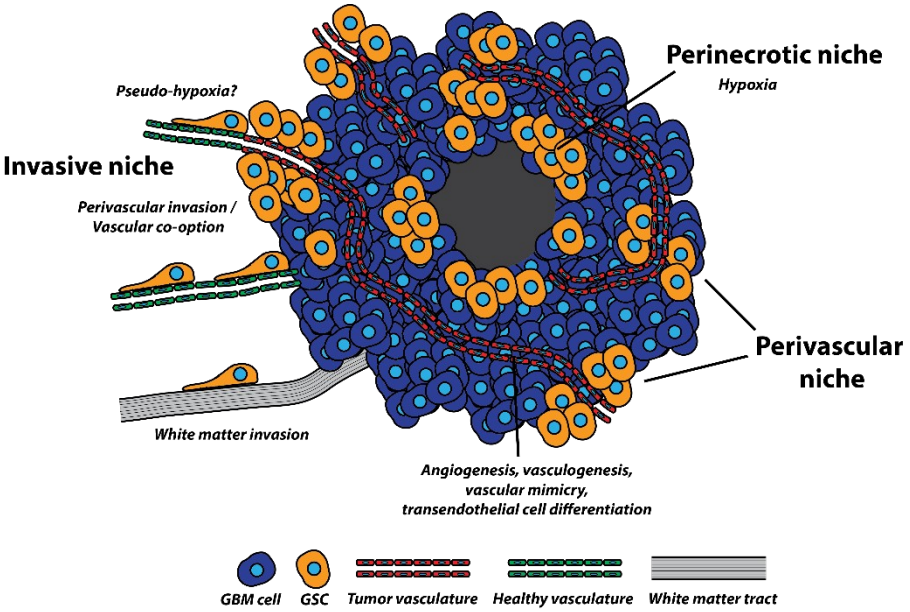

Figure 1.3. *Illustration of the proposed GSC niches; the perinecrotic, perivascular and invasive niche.*

GSCs in brain tissue invasion

GBM tumor formation and progression depends on several key mechanisms, including invasion, angiogenesis and immunosuppression in which GSCs play a crucial role. GSCs mainly utilize two defined routes of invasion; via perivascular spaces and white matter tracts. In perivascular invasion, GSCs are attracted by bradykinin secreted by endothelial cells and have been shown to use different membrane proteins for invasion, such as CXCL12-CXCR4 and Notch-Jagged interactions [34–36]. GSCs in perivascular invasion have been characterized as Nestin+CD133+ [23], indicating a neural stem/progenitor phenotype, whereas another study showed specific perivascular invasion from Olig2+ GSCs via Wnt7 signaling, indicative of an oligodendrocyte progenitor phenotype [37]. The study further displayed that Olig2+ GSCs invaded as single cells in contrast to Olig2- which demonstrated collective cell invasion [37]. In white matter-based invasion, similar mechanisms of GSC-substrate interactions have been found to be applied, i.e. via Notch1-Jagged1 mediated invasion [38]. Myelinated fibers contain the membrane protein Nogo-A which inhibits cell migration via inactivation of RhoA signaling, however, GSCs have found a way to overcome this challenge [39]. Upon encountering Nogo-A, GSCs employ a decoy mechanism by rapid secretion of SPARC which binds Nogo-A and blocks its inhibitory effects on RhoA signaling [39]. Wang et al. characterize the white matter-invading GSCs as mostly CD133+Notch1+ and CD44- [38]. One study has shown that distinct anatomical regions such as the hippocampus have been spared for GBM invasion, perhaps

indicating a preference for certain extracellular matrix (ECM)/substrate compositions [40].

In order to penetrate the healthy brain parenchyma, the GSCs also interact with and degrade healthy ECM via different integrins, matrix metalloproteinases (MMP) and a disintegrin and metalloproteinases (ADAM) [41–43]. For example, FoxM1-controlled expression of ADAM17 activated the EGFR pathways which resulted in a mesenchymal transition and increased migration in GBM cells [44]. Tenascin-C induced ADAM9 activation was also shown to induce invasion in GBM cells, and silencing ADAM9 resulted in less ECM adhesion and cancer cell migration by interference of β1-integrin activity at the plasma membrane [45,46]. The mesenchymal features (i.e. high CD44 expression) often adopted by GBM cells prior to invasion indicates that the invasive cells often possess stem-like characteristics, although proneural GSCs (Olig2+) have also been shown to venture down an invasive path [37,47].

The neovascularization cascade

GBM invasion often occurs in perivascular spaces of healthy blood vessels which can be considered the first step of the tumor neovascularization cascade that occurs in GBM, namely vascular co-option. Vascular co-option is defined as the process of tumor cells surrounding healthy blood vessels, leeching off the oxygen and nutrients [48]. Inevitably, the tumor cells start expanding rapidly in the area around the newly adopted blood vessel generating a dense tumor core [49,50]. With the tumor cell expansion follows endothelial cell apoptosis and blood vessel disintegration, which leads to hypoxia switching on the angiogenic 'switch' in order to form new blood vessels [50,51]. At this point, several different mechanisms of neovascularization have been identified, including angiogenesis, vasculogenesis, vascular mimicry and glioma-endothelial cell transdifferentiation [49]. The most widely studied is the process of angiogenesis which, similar to vasculogenesis, is triggered by local hypoxia which causes GSC secretion of VEGF and SDF-1α and upregulation of endothelial CXCR4 [52,53]. In addition to this, GSCs have been shown to generate tumor-derived pericytes via TGF-β1 to support the functionality of newly formed blood vessels [54]. Upregulation of endothelial CXCR4 attracts CXCL12 on GSCs and thus also promotes invasion [55].

Immuno-manipulation

In order to avoid death by hand of the immune system, GBM cells have acquired the ability to recruit immune cells, such as macrophages and T cells, and manipulate them into an immunosuppressive phenotype via upregulation of several proteins such as TGF-β, IL-6, aryl hydrocarbon receptor, CD39 and PD-L1 [56–58]. Not only peripheral immune cells are affected, but also reactive astrocytes and microglia contribute to the immunosuppressive environment in GBM and thus tumor progression [59]. GBM subtype specific differences in the immune landscape exists with CD68+ microglia and CD163+ BM-derived macrophages being the most prevalent in all subtypes to different extents [60]. Mesenchymal GBM displayed the

highest infiltration of microglia, macrophages and lymphocytes [60]. Responsible for the immune-manipulation, GSCs have been shown to play a key role in attracting immune cells and shaping the immunosuppressive landscape of GBM [61–64].

Several of the pathways mentioned here overlap with the different functions described. For example, ADAM17 has been shown to be involved in CD44-HIF-2α signaling, regulating both matrix modulation and perivascular hypoxia response [29]. Furthermore, Notch1 was involved in both perivascular and white matter invasion, but was also shown to induce an angiogenic phenotype by stimulation of pericyte-like differentiation and upregulation of several angiogenic factors such as heparin binding EGF and VCAM1 [35,38,65]. These examples demonstrate that both pathways and functions dynamically overlap in order to support GBM progression.

1.1.3. A PLASTIC UNIVERSE: ACQUIRED RESISTANCE

In addition to the intercellular heterogeneity another layer of complexity is added to the tumor equation with many of the cells present in tumors being highly dynamic or *plastic*. Plasticity is the ability of a cell to adapt to external factors such as changes in the extracellular environment surrounding the cell or hostile factors such as chemotherapy and irradiation. In cancer, this phenomenon has been suggested as a mechanism for cancer cells to overcome treatments and has been linked to cancer stem-like cells [66,67]. This has been illustrated multiple times in GBM where the recurrent tumor displays a much more aggressive and treatment resistant phenotype than the primary tumor [68]. GBM tumors initially characterized as proneural had shifted towards a mesenchymal phenotype after recurrence, rendering it more resistant to treatment [68,69]. Also in the invasive edge (or niche), GSCs transitioned from a CD133+ proneural signature to a CD109+ mesenchymal signature in response to radiotherapy [70]. On a more macroscopic level, GBM cells were shown to rapidly increase their formation of tumor microtubes in response to standard GBM treatment modalities [71,72]. The GBM cells where able to stabilize their levels of intracellular calcium by increased calcium communication with surrounding cells mediated through Connexin 43 in interconnected tumor microtubes, thus evading cell death [72].

1.2. TUMOR MORPHOGENESIS: DEVELOPMENTAL GENES RE-EXPRESSED

Cellular plasticity is also key during embryonal development where, unlike cancer, coordinated gradients of different growth factors known as morphogens provide concentration-dependent cellular cues to shape the architecture of organisms. The process of organismal development is called morphogenesis and has for centuries to

this day fascinated researchers [73]. Alan Turing was mostly renown for breaking the enigma code and his contribution to computer science, however, after the second world war Turing found an interest in mathematical biology. In 1952, he published a paper on the chemical basis of morphogenesis, in which he presented a mathematical model of chemical diffusion of morphogens ultimately suggesting a chemical gradient that allows for anatomical differentiation [74]. Since then, this theory has been verified experimentally and expanded upon, identifying multiple morphogens in the process and cellular structures responsible for their distribution [75]. Members of the TGFβ family were the first extracellular morphogens to be described in *Drosophila* and *Xenopus* studies [75]. For example, the bone morphogenic protein (BMP) homolog Decapentaplegic was found to pattern the dorsal-ventral axis in *Drosophila* embryos [76]. By injecting RNA transcripts of Decapentaplegic into young embryos, the increased concentration decreased ventral ectoderm development and increased development of the most dorsal tissues [76]. Several cellular mechanisms known to occur during morphogenesis have also been observed in tumors, such as collective cell migration [77]. Countless morphogens have been discovered in various tumor settings as well such as TGFβ, Nodal, Wnt proteins and hedgehog, indicating that tumors utilize morphogens for their development and growth, much like healthy tissue [78–80].

1.2.1. CELLULAR PROTRUSIONS CONVEYING MORPHOGEN DISTRIBUTION AND CELLULAR MIGRATION

Cellular membrane protrusions come in many shapes and sizes and exert many different functions such as sensing the cell's surroundings, cell migration and cell communication [81,82]. The largest of these structures are lamellipodia which are large F-actin rich protrusions at the cell's leading edge, often ruffled in appearance [81,83,84]. In the smaller end, different types of filopodia-like structures have been identified including tunneling nanotubes, retraction fibers, tumor microtubes and specialized filopodia/cytonemes, all of which are also rich in F-actin [72,85–87]. In general, filopodia are small finger-like protrusions of the plasma membrane often extending from lamellipodia at a cell's leading edge. Filopodia are thought to participate in sensing environmental cues and in orchestrating directional cell migration [88]. Tunneling nanotubes are thin, open-ended tubes between cells allowing for free transport of molecules between them, whereas tumor microtubes are similar but with closed ends [89]. In tumor microtubes, the transfer of molecules instead occurs via gap junctions such as complexes of connexin-43 [72]. From the trailing edge, retraction fibers are thin filopodia-like structures that extend rearward and are left behind the cell as it moves, and has been shown to be essential for detachment from adhesive substrates [90–93]. Beneath the cell, short actin-rich protrusions called puncta or podosomes (or invadopodia in malignant cells) mediate adhesion and matrix degradation during cell migration and are typically identified by the co-localization of F-actin and cortactin [94,95]. The cellular membrane protrusions are illustrated in Figure 1.4.

The mechanisms by which morphogenic gradients are formed include free diffusion, cellular transcytosis and via cellular protrusions such as filopodia [86]. Filopodia structures involved in morphogen transport are typically referred to as specialized filopodia, cytonemes or just filopodia, and their structure and composition is highly similar to filopodia discovered in other contexts [75,86,87]. Filopodia-based signaling is highly active during embryogenesis, transporting Wnt proteins to create concentration gradients during tissue patterning (illustrated in Figure 1.5) [96–99]. In order to form and regenerate tissue, cell migration is most often required, including both physiological and malignant processes such as embryogenesis and carcinogenesis. A cell can sense its environmental surroundings, and the subsequent trigger of migration can either be in response to bound (haptotaxis), soluble (chemotaxis) or mechanical (durotaxis) cues [100]. Filopodia have been shown to be implicated in all of these processes; in haptotaxis through ECM adhesion via integrins present at the filopodia tip, chemotaxis through morphogen secretion inducing migration in surrounding cells, and durotaxis by probing ECM topography and rigidity [101].

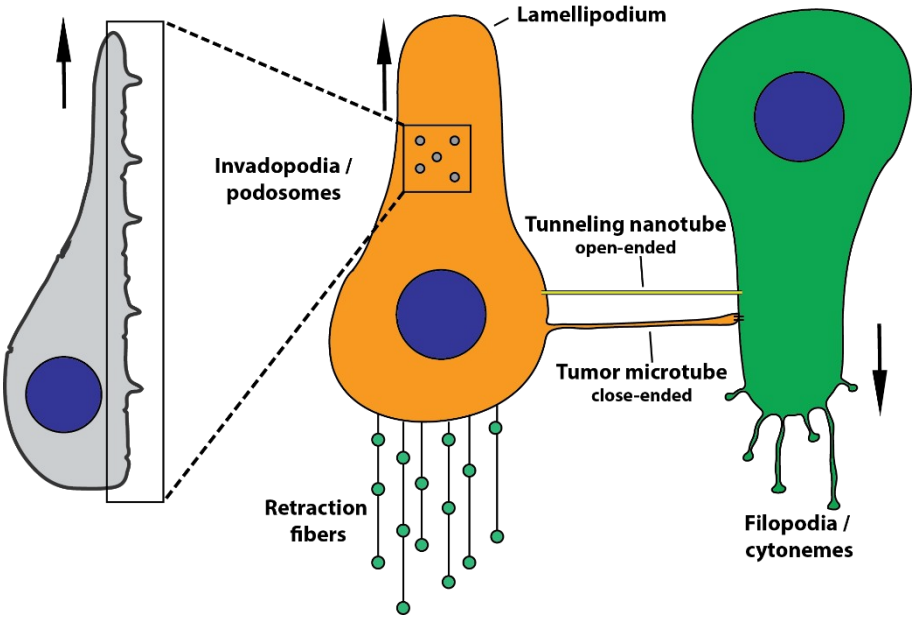

Figure 1.4. *Overview of the different cellular membrane protrusions introduced. Arrows indicate the direction of cell movement.*

1.2.2. MORPHOGENS IN GBM

Cellular membrane protrusions, such as the ones mentioned above, are thought to be present in all cell types in various forms and degrees, including GBM cells [89,102]. Although morphogen transport in membrane protrusions have been scarcely studied

in GBM, several morphogens have been found to play key roles in GBM pathogenesis. The morphogen Wnt5a was shown to be a key driver of the invasive GSCs and by inhibiting Wnt5a expression cell invasion decreased and host survival increased [103,104]. Wnt proteins were also responsible for GBM cell-microglia interaction and activation, ultimately resulting in GBM progression by promoting immunosuppression and cell invasion [105]. During GBM tumorigenesis, the TGFβ-related protein Nodal was observed to be highly expressed in GSCs and downregulated in differentiated GBM cells [106]. In patient-derived GBM cells, dysregulation of the Wnt pathway was shown to be caused by the lack of Wnt pathway inhibition by secreted frizzled-related protein 1 (SFRP1) due to epigenetic silencing of the SFRP1 promoter [107]. Subsequently, treatment with recombinant SFRP1 resulted in decreased tumorsphere formation [107]. TGFβ was also shown to induce and maintain GSC self-renewal through induction of leukemia inhibitory factor expression in patient-derived GBM cells [108]. Sonic hedgehog (Shh), another important morphogen in mammalian development, was shown to initiate tumor formation in neural stem cells when dysregulated in a zebrafish model [109]. As previously mentioned, hypoxia-induced stemness is highly involved in several of the GSC niches and functions that drive GBM progression. During embryonal development, hypoxia also plays a crucial role in regulating cellular differentiation and growth through the same genes as in GBM, such as members of the HIF family [110,111]. These examples clearly underscore a close resemblance between pathways active during morphogenesis and GBM formation/progression. Therefore, targeting morphogen-expressing GBM cells could be a way to enhance treatment efficiency.

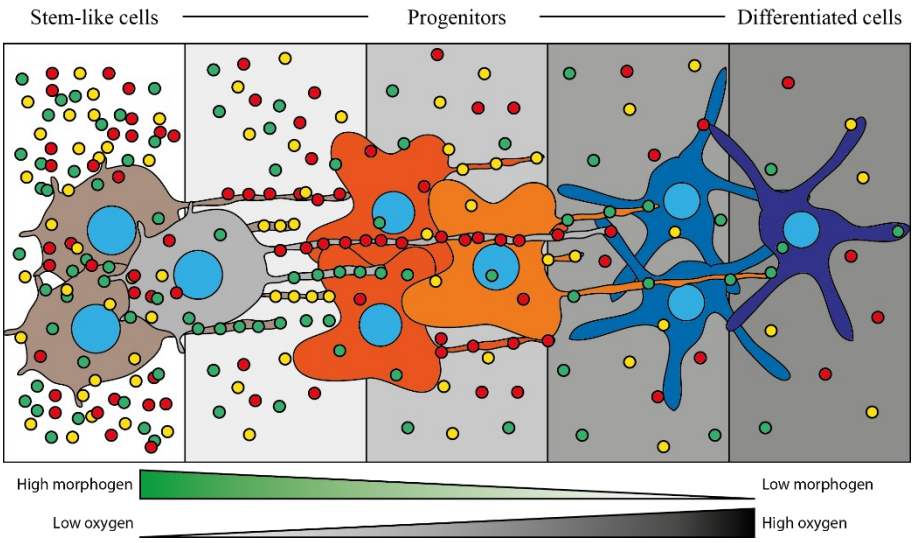

Figure 1.5. *Suggested mechanism of morphogen and oxygen distribution that maintains stemness and induces cellular differentiation in a concentration-dependent manner in GBM.*

1.3. TARGETING THE PLASTIC CELLS IN GBM

Treatment of GBM tumors has proven a monumental task evident by the fact that the disease still is uncurable and only modest improvements have been seen in patient overall survival. As outlined in this introduction, one of the major obstacles of effectively treating GBM tumors is the ability of GSCs to adapt to hostile environments. Thus, targeting the plastic GSCs could be key for future GBM treatments.

1.3.1. CRIPTO-1 AS A POTENTIAL TARGET IN GBM

Cripto-1 is a glycosylphosphatidylinositol (GPI)-anchored surface protein involved in the morphogenic signaling pathways Wnt/β-catenin and TGFβ/Smad (pathways summarized in Figure 6) [112–114]. Cripto-1 is a member of the EGF-CFC domain family of proteins which all contain the evolutionarily conserved EGF and CFC domains [112]. Cripto-1 acts as a co-receptor for several morphogens such as Nodal and Notch, and thus participate in embryogenesis [115]. During embryonal development, Cripto-1 was crucial for tissue polarization during gastrulation responsible for correct orientation of the anterior-posterior axis [116,117]. Moreover, Cripto-1 was involved in the generation of the notochordal plate, prechordal mesoderm and foregut endoderm [118]. In the adult, Cripto-1 expression has been proposed to be turned off or limited to small stem cell populations, only to see it highly re-expressed in several types of cancer [119,120]. Cripto-1 has been associated with processes that relate to its function during embryonal development such as cell invasion or migration, epithelial-mesenchymal transition and treatment resistance, thus seeming important in tumor cell plasticity [121–124]. In GBM, Cripto-1 has not been extensively studied, but has been found in sera and tissue from GBM patients and Cripto-1 expression correlated with shorter survival [125,126]. Studies investigating Cripto-1 overexpressing U87MG cells showed that the cells increased expression of stemness markers Nanog, Oct4, Sox2 and CD44, and that the cells became more proliferative, invasive and angiogenic *in vitro* [127,128]. In support of this, overexpression of Nodal increased Cripto-1 expression *in vitro* and increased glioma progression *in vivo* [129]. Due to its extensive presence shown in stem-like cells in other cancers, several papers have suggested Cripto-1 as a target for therapy [130–132]. However, Cripto-1 knockout studies have shown that it has detrimental impact on mouse embryos, where no Cripto-1 knockout mice were born due to aberrant morphogenesis before and during gastrulation [117,133]. The effects of Cripto-1 inhibition in healthy adults has not, to my knowledge, been investigated and thus side effects from targeting Cripto-1, if functionally inhibited by the treatment, need to be examined. Since GBM has no cure and the median survival is so low, looking into targeting Cripto-1 might still be relevant.

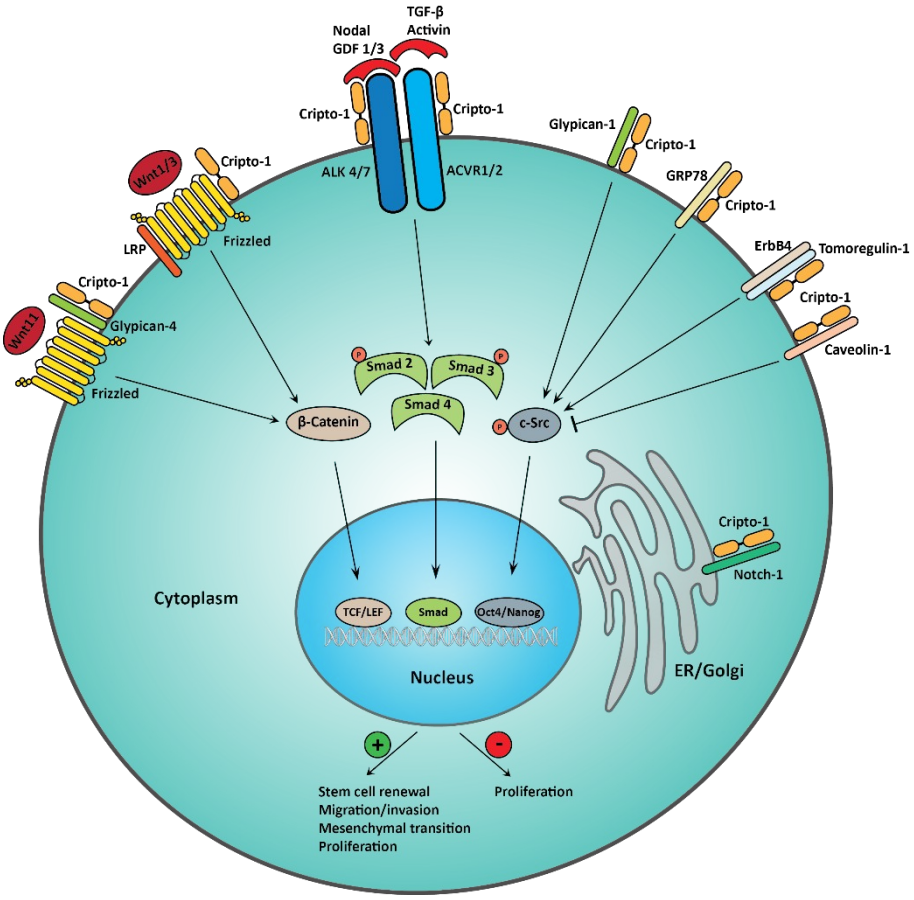

Figure 1.6. *Summary of Cripto-1 interaction partners, pathways and functions.*

1.3.2. CD44 IN GBM

CD44 is associated with stem cells in various healthy and malignant tissue, including GBM [134]. CD44 is a transmembrane glycoprotein consisting of an extracellular domain, transmembrane domain and a cytoplasmic tail, and comes in several different splice variants which have been shown to be involved in different functions [135–138]. Some studies suggest that a soluble form of CD44 exists that is functional and can be used as a plasma biomarker [138–140]. The main ligand of CD44 is hyaluronan (or hyaluronic acid, HA) which is a major constituent of ECM, and further upregulated during processes where cell migration is key such as embryonal development and inflammation [141]. In low-grade glioma (LGG) and GBM patients, CD44 expression has been shown to be increased and linked with a poor overall survival [142–144]. CD44 has been linked to cellular resistance to treatments, for example by upregulating multidrug transporters [145,146]. Although

less investigated in GBM, CD44 expression has been found to contribute to treatment resistance [16,22,147,148]. Depletion of CD44 was shown to cease GBM tumor growth *in vivo* and sensitize the cells to cytotoxic treatment by inhibition of the Hippo signaling pathway [147]. Mesenchymal GBM was shown to be $CD44^{high}OLIG2^{low}$ and was highly resistant to radiation treatment compared to proneural GBM which was defined as $CD44^{low}OLIG^{high}$ [16]. Another study showed that $CD44^{+}$ GSCs residing in the perivascular niche were radioresistant due to activation of CD44 via Osteopontin [22]. This indicates that combining CD44 inhibition with standard GBM treatment could make it more effective, however, side effects related to targeting such a common receptor need to be evaluated carefully.

1.4. CELLULAR MODELS OF GBM

The past decades have demonstrated significant technological advances within fundamental biological research allowing scientists to analyze GBM tumors with ever-increasing resolution. The resulting new knowledge about GBM tumors brought with it a realization concerning the lack of clinical reproducibility of cellular models of GBM, creating a shift from 'generic' GBM cell lines towards patient-derived cells.

1.4.1. HUMAN PATIENT-DERIVED GBM CELLS

The most commonly used GBM cell lines are U87MG and U251, both isolated from patients in the late 1960's [149,150]. The cell lines were selected by adherence to a substrate using serum-rich medium. Three decades later, the fundamental identification and purification of brain tumor stem-like cells using CD133 as a marker solidified the cancer stem cell hypothesis in solid brain tumors [151,152]. These studies employed a protocol for neural stem cell isolation and culture conditions on dissociated brain tumor material resulting in multicellular non-adherent tumorsphere cultures. Such culture media formulations are still used today with slight differences in the supplements added [153,154]. When isolating GSCs, some laboratories have sorted for CD133+ cells in order to further enrich for stem cell properties, whereas most do a 'bulk' isolation without sorting to maintain the original tumor cellular heterogeneity [153,155,156].

Patient-derived GBM cells (PDGCs) are rarely (if ever) cultured under conventional culture conditions with the addition of serum, but rather maintained in GSC culture medium with added growth factors and supplements that support stemness as tumorspheres. In recent years, more research has been put into growing PDGCs as organoids in order to preserve intercellular heterogeneity and further push the limits of the complexity of cell cultures *in vitro*. Here, single GBM cells directly from dissociated tissue or tumorspheres are embedded in a relevant ECM (matrigel, for example) in GSC medium on an orbital shaker in a humidified incubator [157,158]. Hubert et al. showed that mouse xenografts using patient-derived tumor organoids

reflected the patient tumor with a diffuse infiltrative growth pattern whereas tumorspheres isolated from the same patients presented as encapsulated tumors when xenografted [157]. Taken a step further, Linkous et al. generated cerebral organoids from human embryonic stem cells and added to this patient-derived GSCs to more accurately recreate gliomagenesis with host-tumor interactions, even demonstrating the role of tumor microtubes in GSC invasion [159]. Generation GBM organoids is a time-consuming process taking up to 12 months for single GBM organoids to fully mature, but still show more clinical relevance both *in vitro* and *in vivo* compared to other GBM culture methods [157,158].

1.4.2. SYNGENEIC MURINE GBM MODELS

As described earlier, the host immune system plays an essential role in shaping GBM pathogenesis and progression. One major pitfall of patient-derived xenograft models is the use of immunocompromised animals to facilitate xenografting. To include an intact immune system in the study of GBM biology and immunotherapies, mainly two methods have been used to generate syngeneic models; genetically modified or chemically induced. Genetically modified models include genetic engineering and transduction with viral vectors. Viral vectors have been used to cause gain-of-function of oncogenes such as PDGFB, EGFRvIII, activated p21-RAS and activated AKT [160]. Genetically engineered models have also been used to activate EGFRvIII, EGFR, p21-RAS, PI3K, CDK4 and MDM2, and loss-of-function of PTEN, TP53, CDKN2A and RB in different combinations [160–163].

The most widely used syngeneic GBM models are the chemically-induced models GL261 and GL26 which have allowed for the preclinical studies of immunotherapies in GBM [164–166]. These cell lines are typically cultured as adherent monolayers with serum added to the culture medium, but GL261 has previously been cultured in stem-like conditions which induced a CD133+ cell subpopulation [167]. GL261 cells, which have been more extensively characterized than GL26, have been shown to replicate some macroscopic features often seen in human GBM such as central necrosis and neovascularization [50]. However, most of the characterization of these cells has focused on the immune aspects [165,168].

CHAPTER 2. THESIS OBJECTIVES

The invasive nature and inherent and acquired therapeutic resistance of GBM tumors calls for better understanding of GSCs to enable the development of novel treatments. The objectives of this thesis were to identify relevant therapeutic targets through an improved cellular and molecular understanding of GSCs.

The overall hypotheses were:

1. Cripto-1 is present in GSCs and could be a promising target to direct treatment in GBM (Manuscript I and II)
    a. Objective: To characterize Cripto-1 in GBM
2. Patient-derived GBM cells represent a relevant model of GBM for use in target identification and molecular understanding of GBM plasticity (Manuscript III and IV)

Four separate studies were conducted to address the overall objectives. The aims of the studies were:

Manuscript I: To investigate the subcellular localization of cripto-1 in an overexpression model to relate its localization to structures associated with its reported functions.

Manuscript II: To evaluate different cripto-1 antibodies for use in detection of wildtype cripto-1, with the goal of investigating cripto-1 expression and localization in GBM cells and tissue.

Manuscript III: To establish a relevant GBM model that recapitulates key GBM features *in vitro* and *in vivo.*

Manuscript IV: To investigate the GBM cellular response to conventional GBM treatments *in vivo* in order to identify significant changes in surface marker expression associated to treatment resistant stem-like cells.

CHAPTER 3. SUMMARY OF FINDINGS

3.1. MANUSCRIPT I

Cripto-1 localizes to dynamic and shed filopodia associated with cellular migration in glioblastoma cells

Johann Mar Gudbergsson, Meg Duroux

Manuscript published, European Journal of Cell Biology [169]

Background: Before investigating whether cripto-1 could be a beneficial target in treating GBM tumors, more knowledge on the expression and subcellular localization of cripto-1 in GBM cells was desired.

Introduction: Cripto-1 is a membrane-anchored protein involved in cell migration during embryonal development through the morphogenic TGF-β/Smad and Wnt/β-catenin pathways and has been found overexpressed in several types of cancer, including GBM. The subcellular localization and dynamics of cripto-1 is poorly studied, which led us to investigate this further.

Main methods: Cripto-1 was stably overexpressed in U87MG cells. To evaluate the localization of cripto-1, immunocytochemistry and fluorescence-based structured illumination microscopy was performed. In order to study the dynamics of membrane structures, live-cell spinning disk confocal imaging was performed using CellMask Deep Red membrane stain.

Results: We found that cripto-1, when overexpressed in U87MG cells, localized to filopodia structures including tunneling nanotubes, but not invadopodial puncta. Cripto-1 positive filopodia were shed from the trailing edge of the cells, which are also called retraction fibers. Furthermore, cripto-1 was present in shorter filopodia structures that resembled vesicles attached to the cell membrane via a lipid and actin rich string. With live-cell confocal imaging we found that these structures were highly dynamic membrane protrusions that could interact with retraction fibers.

Conclusion: We conclude that cripto-1 localized strongly to filopodia and retraction fibers, which indicates that the reported function of cripto-1 harmonizes with its subcellular localization found in this study.

3.2. MANUSCRIPT II

An evaluation of different Cripto‐1 antibodies and their variable results

Johann Mar Gudbergsson, Meg Duroux

Manuscript published, Journal of Cellular Biochemistry (2019) [170]

Background: Continuing our investigation of cripto-1 in GBM from Manuscript I, we wanted to detect cripto-1 in patient-derived GBM cells under wildtype conditions (not overexpressed).

Introduction: Antibody-based detection of cripto-1 is widely used in cripto-1 research. High plasma/serum cripto-1 and immunohistochemistry detection of cripto-1 has been found to correlate with worse prognosis in several cancer studies. In this study, we present a concise systematic review of cripto-1 antibodies and an experimental evaluation of four widely used cripto-1 antibodies.

Main methods: Primary GBM cells were cultured in serum-free conditions and seeded on geltrex matrix to study invasive cells. Immunocytochemistry was used to detect cripto-1 using four different anti-cripto-1 antibodies. All four cripto-1 antibodies were tested for binding to NTERA2 cells fixed with formaldehyde or methanol. A systematic literature review was performed on cripto-1 antibodies. Lastly, sequence alignment (pBLAST) and 3D-Match structural comparisons (Phyre2) were used to compare preserved protein domains of cripto-1.

Results: We found a non-specific binding of a cripto-1 antibody to the geltrex matrix onto which primary GBM cells were seeded, and no binding to cells. We thus tested four different cripto-1 antibodies, and found that they all, in varying degree, bound to geltrex. To further test how the antibodies bind, we bought the NTERA2 cell line as it is generally used as cripto-1 positive control. All four cripto-1 antibodies bound differently to NTERA2 cells, with one antibody even showing intracellular filament staining. We then did a systematic review of cripto-1 antibodies in order to map the different epitopes or domains that the antibodies were designed to bind on cripto-1. Here, we found that many cripto-1 antibodies were designed to bind to highly conserved domains within cripto-1, namely the EGF- and CFC-domain. Lastly, we highlight some possible cross reactants from similar domains contained in other proteins.

Conclusion: We conclude that the different cripto-1 antibodies investigated here present highly variable results and require more detailed characterization in future cripto-1 studies.

3.3. MANUSCRIPT III

A tumorsphere model of glioblastoma multiforme with intratumoral heterogeneity for quantitative analysis of cellular migration and drug response

Johann Mar Gudbergsson, Serhii Kostrikov, Kasper Bendix Johnsen, Frederikke Petrine Fliedner, Christian Brøgger Stolberg, Nanna Humle, Anders Elias Hansen, Bjarne Winther Kristensen, Gunna Christiansen, Andreas Kjær, Thomas Lars Andresen, Meg Duroux

Manuscript published, Experimental Cell Research (2019) [171]

Background: In order to identify GBM targets in future studies, we needed a well-characterized primary GBM cell model for *in vitro* and *in vivo* studies.

Introduction: Due to the poor overall survival in GBM patients, novel treatments need to be developed to target the invasive cells. Often drug screening and drug response assays are performed on generic GBM cell lines which could undermine the translational potential.

Main methods: Patient-derived primary GBM cells T78 were cultured as tumorspheres in serum-free conditions. Tumorsphere migration assay was performed by seeding single tumorsphere onto geltrex matrix. For drug response evaluation, extracellular vesicles and oxaliplatin were tested. Migration was tracked using phase-contrast time-lapse imaging and area quantification. Immunofluorescence imaging on cells was performed with a structured illumination microscope and immunohistochemistry with a fluorescent slide scanner.

Results: Differential intercellular expression of GFAP and Nestin was found in tumorspheres seeded onto geltrex with $GFAP^-$ $Nestin^+$ cells primarily located in the migrating cells in the tumorsphere periphery. We showed that this expression pattern was also present *in vivo*. We furthermore established that our geltrex migration assay with patient-derived GBM cells was suitable to test cancer growth inhibitors and stimulators illustrated with oxaliplatin and extracellular vesicles, respectively.

Conclusion: The geltrex tumorsphere migration assay with patient-derived GBM cells showed location-based intercellular heterogeneity and could be used for drug response evaluation *in vitro.*

3.3.1. DIFFERENCE IN GBM TUMOR GROWTH BETWEEN U87MG AND PATIENT-DERIVED T78 CELLS

Prior to the studies conducted in Manuscript III, we performed a pilot study in order to compare the growth curves of the widely used generic cell line U87MG and the patient-derived T78 cells. GBM cells were implanted as described in Manuscript III, and tumor growth was monitored with MRI. U87MG tumors grew to a mean size of 31 mm^3 in 23 days compared to a mean size of around 10 mm^3 in 49 days with T78 tumors (Figure 3.1). Although growing a lot slower than U87MG tumors, T78 tumors showed a steady growth with less inter-tumor size variance.

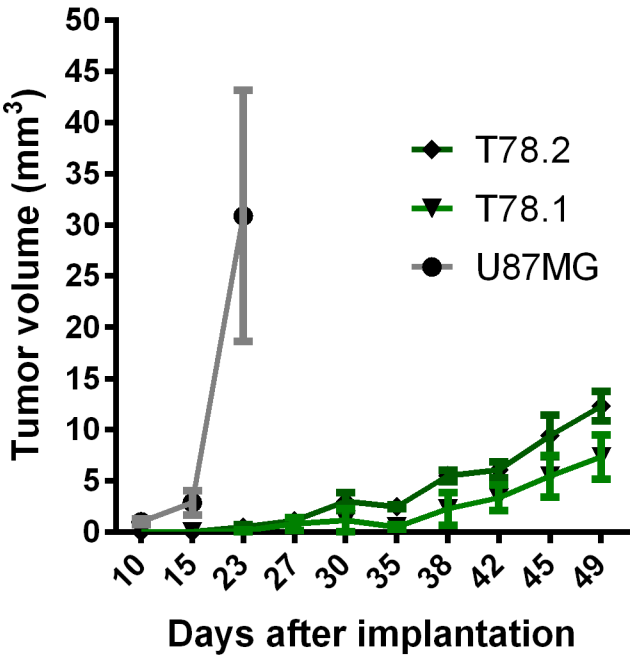

Figure 3.7*. U87MG & T78 tumor growth curves. U87MG GBM cells (200.000 per mouse, n = 3) and patient-derived T78 GBM cells (100.000 or 200.000 per mouse, T78.1 and T78.2, respectively. n = 3) were implanted into NMRI nude mice and monitored with MRI as described in Gudbergsson et al. 2019 [171]. Data are presented with mean ± SD.*

3.4. MANUSCRIPT IV

Conventional treatment for glioblastoma reveals persistent CD44+ subpopulations

Johann Mar Gudbergsson, Esben Christensen, Serhii Kostrikov, Kasper Bendix Johnsen, Torben Moos, Meg Duroux, Andreas Kjær, Thomas Lars Andresen

Manuscript submitted

Background: Expanding on the study presented in manuscript III, we wanted to use the model to characterize drug response *in vivo*.

Introduction: In GBM, treatment resistance represents a tremendous problem. Understanding how GBM cells respond to conventional GBM treatments could reveal new potential targets for novel therapies.

Main methods: Intracranial xenografts of patient-derived T78 cells were performed in NMRI nude mice, which were monitored weekly with MRI for tumor growth. Irradiation, temozolomide or combination treatments were administered once tumors averaged 5 mm^3. Flow cytometry was performed on single cell suspension from dissociated mouse brains, and subsequently analyzed with FlowSOM cluster analysis. Immunohistochemistry and fluorescence microscopy were performed for qualitative analyses. TCGA patient analyses were done with the OncoLNC online tool.

Results: Treatments significantly reduced tumors without eradicating them, leaving behind GBM cells that could be analyzed with flow cytometry for the expression of CD34, CD44, CD133 and CXCR4. Most significantly, an approximate 8-fold increase in CD44+ GBM cells was seen in all treatment groups. This was confirmed by cluster analysis that showed a relative increase in several CD44+ subpopulations, including one that was $CD44^+$ $CD133^-$ $CXCR4^-$ $CD34^-$. Immunohistochemistry results cemented these findings and revealed a mouse-derived $CD44^+$ response around GBM tumor cells. $CD44^+$ and $Nestin^+$ GBM cells were furthermore shown to be invasive and residing in perivascular niches, and irradiated GBM cells displayed increased tumor microtubes. Lastly, GBM and low-grade glioma patients with high expression of CD44 had a decreased overall survival compared to patients with low expression.

Conclusion: We show here that CD44 is involved in the cellular response to conventional GBM treatment in patient-derived GBM cells, and could be of importance in identifying and targeting potential treatment resistant cells in GBM patients.

CHAPTER 4. GENERAL DISCUSSION

This discussion will focus on putting all the manuscripts in a coherent meta context, and thus will each of the discussions from the studies not be repeated unless for contextual purposes. Overall, I will here present a reflection of fundamental concepts relating to GBM biology and the targeting of GSCs.

4.1. THE FUTURE OF CRIPTO-1: POTENTIAL AND PITFALLS

Cripto-1 has been shown to play an important role during embryonal development and in malignant progression in several types of tumors. The attractive notion that Cripto-1 is only expressed at low levels in the adult and seemingly highly expressed in tumors makes it a potential target for treatment. It has been implicated in processes that are hallmarks for cancer stem or progenitor cells, perhaps acting in a morphogen-like fashion as proposed during embryonal development. In Manuscript I, we detailed the subcellular localization of Cripto-1 to filopodia structures and saw it being shed in lipid-rich structures (shed filopodia or retraction fibers) and present in dynamic, plasma membrane-attached filopodia [169,172]. The localization to filopodia structures fits well with Cripto-1 being a morphogen-associated protein since filopodia are implicated in generating morphogen gradients. However, when looking for wildtype Cripto-1 expression in primary GBM cells, we saw that four different Cripto-1 antibodies yielded different results (Manuscript II). This raises concerns since many studies that have discovered Cripto-1 in the different cancers use anti-Cripto-1 antibodies to reach their conclusions. Only two studies have investigated and found Cripto-1 in GBM and are also using anti-Cripto-1 antibodies to reach their conclusions [125,126]. Tysnes et al. use the polyclonal 600-401-997 from Rockland to investigate Cripto-1 as a prognostic biomarker in GBM [125]. This antibody was a part of our evaluation in Manuscript II and yielded different results from the other three antibodies evaluated. Pilgaard et al. use a few different anti-Cripto-1 antibodies for the different methods used to examine Cripto-1 expression. For western blotting of wildtype U87 cells they used a goat anti-human polyclonal antibody from R&D Systems showing an intense Cripto-1 signal just below the 37 kDa mark [126]. We did anti-Cripto-1 western blots in Manuscript I with the ABD13 antibody, showing no Cripto-1 binding to wildtype U87 cells. In the Cripto-1 overexpressing U87 cells, we showed Cripto-1-positive bands around the 25 kDa mark. This substantiates the concerns raised about Cripto-1 antibodies and could call into question whether Cripto-1 is expressed in GBM.

So, how can we in Manuscript I then trust the results when the antibody used is questioned in Manuscript II? The antibody used in Manuscript I (ABD13) does not stain wildtype U87 cells in our hands but does so after transfection with a Cripto-1 plasmid. This was shown with western blotting and fluorescence microscopy using

ABD13 and flow cytometry using FAB2772P anti-Cripto-1 antibodies. Instead of using antibody-based protein detection, we could have investigated transcriptional levels of Cripto-1 with qRT-PCR or *in situ*-hybridization methods. For antibody-independent protein detection, mass spectrometry could have been used. However, to identify whether Cripto-1 is present in GSCs, it is necessary to know whether the Cripto-1 expressing cells are GSCs or not, and thus simultaneously needs to be evaluated for the presence of GSC markers. Furthermore, to investigate the anatomical location of Cripto-1+ (i.e. in the different GSC niches) microscopy techniques are required, in which the use of antibodies is desired.

To confidently investigate wildtype Cripto-1 protein expression in GBM, better characterization of current Cripto-1 antibodies needs to be done and new well-characterized monoclonal antibodies needs to be developed. After raising the concern on Cripto-1 antibodies we decided not to put more work into investigating the potential of Cripto-1 as a target in GBM.

4.2. CD44 AS A POTENTIAL TARGET

In order to identify a new potential GSC target, we used our previously described model (Manuscript III) and conducted a larger *in vivo* study looking at how the GBM cells respond to TMZ, IR and TMZ+IR (Manuscript IV). In GBM, CD44 has been found residing in several GSC niches, such as the perivascular and peri-necrotic niche [22,142,173]. CD44 was also found expressed in GSCs and abundant expression in the treatment resistant mesenchymal GBM cells [16,147,174]. In Manuscript IV, we observed an increase in CD44+ cell populations as a result of treatment, and an increase in stromal mouse-derived CD44 was seen expressed in and around the tumor indicating a reactive CD44 host-response to the tumor. Other studies have also found upregulation of CD44 in response to irradiation treatment in GBM models [70]. Since CD44 expression seems to be upregulated in GSCs and represents the most aggressive GBM cells, it could be a potential target in GBM treatment. Although CD44 has mostly been described in cancer settings, it has also been implicated in embryo and tissue development (or morphogenesis) [175–178]. In Manuscript I, we saw that CD44 co-localized with Cripto-1 in dynamic and shed filopodia, perhaps indicating that CD44 could be involved in some of the same processes as Cripto-1. The notion of CD44 being a morphogen-associated protein only strengthens its potential as a possible target in GBM. As seen in Manuscript IV, the added upregulation of CD44 in the stroma around tumors combined with high expression in GSCs could further increase the directed uptake of CD44-targeted treatment [179,180]. However, CD44 is a highly complex protein consisting of several isoforms that have different functions. In GBM, not much research has been put into elucidating the expression of the different variants but one study found that CD44v6 in particular regulates GSC growth [181]. This variant has also been found to be expressed in CSCs in other tumor types, especially those of hematopoietic or epithelial origin [182,183]. Targeting of CD44s and CD44v6 has been done in a few

studies in rodent pancreatic cancer models *in vivo* which inhibited tumor growth [184,185]. Side effects were, however, not evaluated.

The expression of CD44 in various normal adult tissue throughout the body, including the CNS, complicates its use as a target and requires rigorous assessment of potential side effects [138,186]. If a functional inhibition of CD44 is sought to be achieved through targeting of CD44, further research into the expression of CD44 variants in GBM could prove fundamental in order to minimize side effects. Otherwise, targeting moieties that do not functionally interfere with CD44 function could be designed in order to circumvent this issue.

4.3. THE ENIGMA OF GSC TARGETING

One thing is to find a potential target and another to develop a suitable treatment approach that will effectively reach the target, especially when that target is protected by the blood-brain barrier (BBB). Several different treatment approaches exist including small molecule-based drugs, nanoparticle drug delivery vehicles such as liposomes or polymer nanoparticles, immunotherapies such as antibodies or antibody fragments, or cell-based treatments such as vaccines or T cell therapies. Being in the brain, GBM is subjected to the protective BBB, but the enhanced permeability and retention (EPR) effect is present in the tumor bulk where tumor-derived vasculature has been formed [187]. During surgical resection, however, most of these blood vessels (if not all) are removed with the tumor bulk and therefore also the tumor cells that are affected by the EPR. The tumor cells still present in the brain in the invasive niche and potential small satellite tumors that have not yet produced their own blood vessels, are most likely still protected by the BBB [188]. This means that any future treatment targeting these cells needs to have the ability to penetrate the BBB and reach the target cells located in the brain, which so far has proven to be a difficult task [189]. Or, in order to bypass the intact BBB, intrathecal administration could be done [190].

When it comes to GSC niches, hypoxia is frequently mentioned as a contributor to niche maintenance whether it being in the perinecrotic or perivascular niche, and one can even imagine that hypoxia could play a role in the invasive niche [174,191]. Thus, even i.v. injection of BBB-penetrable drugs might not effectively reach the GSCs in hypoxic niches since the distance from a blood vessel could be too far to rely on diffusion of the drug. In contrast, intrathecal delivery of IgG antibodies in healthy rats showed distribution throughout the whole brain in perivascular spaces of all vessel types, which was further enhanced if co-administered with hyperosmolar mannitol [190]. This could indicate that intrathecal administration could deliver treatment to GSCs residing in the perivascular niche. Since neurons and white matter tracts are rarely more than 10-20 μm from capillaries (within the range of diffusion), even the invasive niche could be targeted [192]. That is, if the diffusion properties in GBM tumors are the same as in the healthy brain – which they are not. In GBM, an increased interstitial pressure and peritumoral edema is often present due to abnormal vasculature and insufficient

lymphatic drainage [193–196]. A failure to maintain pressure differences between capillaries and brain parenchyma could result in capillary stasis which could similarly affect the perivascular flow in those regions. In such cases, perivascular diffusion could be limited, thus resulting in inefficient targeting of invasive GSCs. Furthermore, in the region of peritumoral edema, Engelhorn et al. found that invasive GBM cells were residing herein [197]. If the peritumoral edema causes less capillary exchange in the invasive region, a hypoxic state could be generated, further contributing to the maintenance of stemness in invading GBM cells. The issues of access to the invasive cells along with their abilities to adapt to treatment, makes it immensely difficult to cure GBM [198–200].

4.4. 'ALL MODELS ARE WRONG, BUT SOME ARE USEFUL'

To be able to further our understanding of GBM tumor biology to progress the development of novel GBM treatments, clinically relevant model systems are key. During the past decades, a vast improvement of cellular and xenograft GBM models has taken place. The isolation of primary (patient-derived) GBM cells has gained increased attention and the methods of propagating the cells have improved, switching to either serum-free non-adherent tumorsphere culture conditions that promote stem-like properties or subcutaneous propagation *in vivo*. Orthotopic intracranial implantation of GBM cells has also seen an increased favoring among researchers compared to subcutaneous GBM tumors, cementing the acknowledgement of the brain milieu in creating a more accurate recapitulation of the clinical GBM picture. Here, I will discuss the GBM model used in our studies and outline and discuss some conceptual challenges that preclinical GBM models face.

In Manuscript III and IV we used the patient-derived GBM model T78, which has previously been characterized *in vitro* and *in vivo* with respect to invasive properties [201–203]. We have added to their characterization some depth *in vitro* by looking into the intercellular heterogeneity based on cellular localization in a geltrex-based tumorsphere migration assay [171]. Here, we showed that cells in the tumorsphere periphery (actively migrating) lost GFAP expression and gained nestin expression. Furthermore, we added dimensions to the treatment side of the tumorsphere migration assay by demonstrating that migration could be increased and decreased with external stimuli [171]. *In vivo*, Munthe et al. did a comprehensive characterization of several stemness markers in several patient-derived GBM cells and found that the peripheral GBM cells contained less stemness markers than in the tumor core (presented as tumor area fraction in %) [202]. In our *in vivo* studies, we saw that all T78 cells were nestin+ when stained with two different nestin antibodies, but GFAP expression was lost in migrating cells in the periphery [171]. Munthe et al. did not co-stain with GFAP, hence we do not know whether the it would be similar in their setup. In Manuscript IV, we expanded on the *in vivo* drug

response characterization of T78 and analyzed how treatment affected GSC subpopulations, illustrating that this model is suitable for such studies.

One major pitfall that generally applies to all tumor xenograft experiments is the lack of or altered host animal adaptive immune system, a necessity for human-derived GBM tumors to form in other species. GBM tumors have long been known to closely interact with the immune system and even harness or manipulate it to their own benefit [204,205]. The immunodeficiency also means that a part of the survival pressure is missing on the xenografted tumor cells which could impact the clinical relevance of a preclinical study, and does not allow studies of cancer immunotherapies such as vaccines or cell-based treatments [206,207]. In Manuscript III and IV we used NMRI nude mice which are convenient to work with for surgical procedures due to the lack of fur. However, our *in vivo* model animal was also immunodeficient and thus subjected to the general pitfalls mentioned here. To circumvent this, researchers have come up with generating several different immunocompetent GBM tumor models, which include spontaneously or chemically induced tumor formation and subsequent cell line generation, genetically engineered models (GEM) and humanized mice [160,208]. While the spontaneous and chemically induced cell lines are syngeneic, they are often poorly characterized with respect to molecular GBM features [50,168]. Mostly, the characterization is based on histology to see if macro-anatomical similarities are present such as angiogenesis and necrosis, making it difficult to directly translate the tumor response to treatment. These models have, however, been used in several preclinical immunotherapeutic assessments that have led to clinical trials [165]. GEMs are produced through a series of mutations in mice which then leads to formation of a syngeneic tumor, and have been shown to be highly controllable and reproducible [160,209]. Although allowing for the study of single proteins or pathways in gliomagenesis, the models could suffer from being too simple when evaluating a treatment response by only representing few features of the tumor of origin. The murine immune system has been heavily used as a tool to understand the human immune system, but due to obvious species differences the two systems might not react in the same way to a tumor or tumor treatment [210–213]. To work around that, humanized mice have been generated by engrafting human hematopoietic stem and progenitor cells into severely immunocompromised mice resulting in a functioning human immune system mixed with the remainder of the murine immune system (strain dependent) [208]. Such models are, however, highly expensive and time-consuming, limiting their present use in research.

The most widely used mouse models are the immunocompromised mouse models, for which the most widely used GBM cell lines for xenograft studies are U87MG and U251, which have been used in more than 2000 and 1000 publications, respectively [214]. These cells are notorious for being easy to culture, have a high xenograft success rate and rapidly produce tumors *in vivo*. Generally, how complex the xenograft tumor is and how well it resembles the clinical picture highly depends

on the quality and integrity of the GBM tissue from which the cells are initially extracted, the method of cell isolation and propagation (for example, adherent vs. tumorsphere), and where they are implanted (orthotopic vs. heterotopic). The logistics and economy surrounding animal experiments might also impact the level of tumor model quality, forcing low-budget laboratories to use generic cell lines instead of primary cells. Two success criteria for a tumor model are, in many cases, how fast the tumor is generated and the inter-animal success-rate of tumor formation. For U87MG and U251 cells, tumors form in almost all inoculated animals and grow to a "sufficient" size within two to three weeks, keeping the turnover time for experiments, and hence also the cost of the experiments, at a minimum [214]. To illustrate how large the difference can be between fast-growing U87MG xenograft tumors and "slow-growing" patient-derived GBM xenograft tumors, we included results comparing U87MG tumor growth to the patient-derived GBM tumor T78 (Figure 3.1). The immense difference sparked a few fundamental questions: Does the time of growth in any way reflect the cellular complexity of the tumors? We did not answer that question experimentally, but in Manuscript III we saw that the T78 GBM tumors were not encapsulated such as is usually seen with U87MG tumors, but were rather well integrated into the mouse brain [171,214,215]. The exponential growth rate in xenografts with generic cell lines such as U87MG and U251 might imply that the majority of cells are actively proliferating. Is that expected in human tumors as well, or could it insinuate intercellular homogeneity? A study by Stensjøen et al. analyzed growth dynamics of 106 GBM patients using MRI and found a mean daily tumor growth of 1.4 % and a mean doubling time of tumor volume of 49.6 days [216]. Comparing these numbers to our preclinical observations in Figure 3.3.

1, we find U87MG with a mean daily growth of 133 % and 18.5 % for T78. This corresponds to a daily growth of U87MG being approximately 100-fold compared to what Stensjøen et al. reported in human GBM patients, and T78 displays a 13-fold difference in daily growth rate [217]. A difference in host-contributed growth dynamics between mouse and human is of course expected, but perhaps the fastest and most consistently growing preclinical GBM tumor models aren't the better choice when it comes to replicating the clinical picture, and thus might be less likely to reflect a treatment response as would be seen in GBM patients.

Intercellular heterogeneity is a clinical hallmark in GBM which should ideally be replicated in preclinical models. If the cells used for xenografting are homogeneous before xenografting the tumor that forms would most likely be homogeneous as well, unless if using GBM stem- or progenitor cells whose cellular fate has not yet been locked in. When it comes to comparison at the molecular level, GBM xenograft tumors formed from freshly isolated patient-derived GBM cells were shown to reflect the morphology and molecular characteristics of the original patient

tumor, both with respect to the molecular subtype classification and expression of Sox2 and EGFR [218–220]. How the cells are cultured after cell isolation from patient specimens has been shown to affect the intercellular heterogeneity and success of integration into host tissue in the resulting xenograft. Hubert et al. were the first to demonstrate that GBM-derived organoids cultured for approximately a year still resembled the patient tumor of origin when xenografted and showed a highly diffuse pattern compared to xenografts derived from a tumorsphere culture of the same cells [157]. Although the tumorsphere culture-derived xenograft in their study produces a highly encapsulated tumor, we showed in manuscript III that diffuse xenograft tumors could be produced from tumorsphere cultures that have been cultured for several passages [157,171].

4.4.1. MODELING THE ROOT OF RECURRENCE: THE 'INVASIVE NICHE'

Throughout this thesis, many different characteristics of invasive GBM cells have been presented. In summary, invasive GSCs are found in invasive niches in the periphery of GBM tumors but are also found in perivascular niches due to vascular co-option (the initial step in the neovascularization cascade) being an invasive process. In order to invade healthy brain parenchyma, the GSCs dynamically regulate different membrane protrusions (such as lamellipodia and filopodia) for anchoring to ECM (haptotaxis), manipulating local healthy/tumor-associated cells via tunneling nanotubes, tumor microtubes and filopodia-dependent morphogen secretion, and for degradation of ECM via invadopodia. On a molecular level, the invasive GSCs often express a mesenchymal phenotype characterized by mesenchymal markers such as CD44, CD109, Notch1 and CXCL12 but have also been shown to display proneural phenotypic markers such as Nestin and CD133, and oligodendrocytic markers such as Olig2. General stemness markers such as Sox2, Oct4 and L1CAM have also been described as expressed in invading GSC [13]. The regulation of the GSCs has often been attributed to the local oxygen concentration where hypoxia serves as a main driver of stemness and invasion through HIF proteins. Being highly plastic cells, GSCs are in general regulated by their local environment which also includes responding to treatment, in which a mesenchymal and invasive phenotype is induced rendering the GSCs more resistant to treatment.

Much research has been put into understanding the invasive cells of GBM that drive its recurrence after heavy first-line treatments, but most of what is known about these cells stems from cultured cells or animal experiments [196]. Maximum safe resection surgery of GBM tumors is generally done with MR-based guidance using gadolinium as a contrast or by using 5-ala fluorescence-guided surgery [221]. These surgery techniques have been shown to not be sensitive enough to detect invasive GBM cells, hence the universal recurrence of the tumor [222]. The only way to characterize these invasive GBM cells would be to examine post-mortem brains from GBM patients. Such studies exist, but only one study using IHC to characterize the invasive cells could be found (with the applied search terms).

Peng et al. examined two post-mortem brains from GBM patients and found that the invasive tumor regions had more CD133 and Sox2-positive cells than other parts of the tumors [223]. With such scarce knowledge of how the invasive cells look at a molecular level in human patients, it might be difficult to translate findings from preclinical studies. Although difficult and expensive, it might be the only way to evaluate the relevance of preclinical GBM models with respect to GSC invasion.

CHAPTER 5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

GBM treatment has seen little progression in the past many years due to its delicate location, invasive nature and heterogenic composition, unavoidably resulting in tumor regrowth. The blood-brain barrier constitutes a significant obstacle in treating GBM tumors, shielding invasive GBM cells from the treatment. In this thesis, a search for potential targets on the surface of hard-to-reach GSCs has been presented in four studies in order to characterize the cells and find ways to direct treatment.

Cripto-1 was hypothesized to be a potential target on GSCs due to its stem cell-associated nature in health and cancer stem cell association in several different types of cancer. We show for the first time a subcellular mapping of cripto-1 in cripto-1 overexpressed U87MG cells and find it highly enriched in filopodia structures, both static/shed filopodia and highly dynamic membrane-anchored filopodia. These findings support the role of cripto-1 in cellular migration. However, in order to specifically detect cripto-1 in GBM tumors or GSCs, cripto-1 antibodies need better characterization to avoid potential false-positive detection. Production of highly specific monoclonal antibodies designed to bind outside of the signal peptide, EGF and CFC domains could facilitate proper detection of cripto-1 in future studies, both as a biomarker and to study wildtype cripto-1 biology.

To enable detection of relevant surface targets on GSCs a model of GBM was developed and characterized *in vitro* and *in vivo*. Here, we found that GBM characteristics seen *in vivo* were similarly recapitulated in *in vitro* tumorspheres seeded on complex ECM such as geltrex. Expanding on this model with a large *in vivo* study, we found that the cellular composition of the GBM tumor changed in the mice that received conventional GBM treatment. Especially one protein was particularly upregulated, namely CD44, which could potentially serve as a target in directed GBM treatment in the future.

In order to improve GBM treatment more fundamental research into the basic biology of GBM is needed, with emphasis on the invading GBM cells that are not affected by surgery and are more resistant to current treatment. This would require more high-resolution work, such as multi-color flow cytometry as we have presented here, on patient biopsies and surgical specimen. A crucial step towards understanding the invasive GBM cells, and how to reach them, is to analyze post-mortem brains with immunohistochemistry techniques.

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