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Hypoxia- and Radiotherapy-Induced Modulation of the Glioblastoma Microenvironment

Rosberg, Rebecca

2026

Document Version:

Publisher's PDF, also known as Version of record

[Link to publication](#)

Citation for published version (APA):

Rosberg, R. (2026). *Hypoxia- and Radiotherapy-Induced Modulation of the Glioblastoma Microenvironment*. [Doctoral Thesis (compilation), Department of Laboratory Medicine]. Lund University, Faculty of Medicine.

Total number of authors:

1

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Hypoxia- and Radiotherapy-Induced Modulation of the Glioblastoma Microenvironment

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DEPARTMENT OF LABORATORY MEDICINE | FACULTY OF MEDICINE | LUND UNIVERSITY



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Rebecca Rosberg



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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on the 23rd of April at 09.00 in Auditoriet, Medicon Village, Lund

Faculty opponent

Monica Olcina, PhD, University of Oxford, Oxford, United Kingdom

Organization: LUND UNIVERSITY

Document name: Doctoral thesis

Date of issue: 2026-04-23

Author(s): Rebecca Rosberg

Sponsoring organization:

Title and subtitle: Hypoxia- and Radiotherapy-Induced Modulation of the Glioblastoma Microenvironment

Abstract: Glioblastoma represents the most aggressive primary brain tumor in adults. Despite intense treatment with surgery and chemoradiotherapy, treatment resistant tumors inevitably recur in close proximity to the primary lesion. Thus, underscoring the need for new therapeutic approaches. Glioblastomas are highly heterogenous tumors, displaying various of molecular and phenotypic properties apart from consisting of a wide range of other cells, such as stromal and immune cells.

The aim of this thesis was to investigate how the non-malignant components of the tumor microenvironment contribute to tumor regrowth. The impact of radiation and hypoxia on non-tumoral cells remain poorly understood, even though these cells reside with tumor cells in the same areas and are exposed to the same treatment. Here, we used a murine GBM model to perform single cell RNA-sequencing and spatial transcriptomics, together with publicly available human data to get further insight to the changes in the tumor microenvironment. We performed *in vitro* functional studies using primary stromal and tumor cells to evaluate possible tumor-driving mechanisms, and finally, confirmed some of these findings in pre-clinical settings. In paper I, we showed that radiotherapy caused both tumor and stromal cells to converge toward mesenchymal and reactive-astrocyte-like phenotypes. This AC/MES shift was tightly linked to high injury-response programs, fitting well with several conceptual frameworks that OPC/NPC drive towards AC/MES-states and a developmental-injury continuum. This transition appeared to be driven in part by highly reactive astrocytes with strong ECM-remodeling interactions with tumor cells. In paper II, we explored whether radiotherapy-induced stromal reactivity could be pharmacologically reversed using a drug-repurposing approach. We identified a compound that modulated the reactive extracellular matrix after radiotherapy resulting in reduced glial-scar formation. In paper III we focused on hypoxic niches and innate immune reactions. We showed that hypoxia and complement activation converge to promote tumor progression by immunosuppression. Paper IV showed that hypoxia induces a pronounced reactive shift of astrocytes, and that their altered ECM expression promotes GBM survival, aggressiveness and stemness.

Overall, this thesis provided further insights into the complex glioblastoma microenvironment, where both radiotherapy and hypoxia increase stromal-cell mediated tumor progression and radioresistance, which provide insights to possible feature target treatments.

Key words: Glioblastoma, astrocytes, hypoxia, radiotherapy, complement system

Language: English

Number of pages: 99

ISSN and key title: 1652-8220

ISBN: 978-91-8021-853-5

Recipient's notes

Price

Security classification

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Rebecca Rosberg



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Paper 3 © 2024 JCI Insight

Paper 4 © 2021 Cells

Cover image by Rebecca Rosberg

Published by:

Department of Laboratory Medicine

Faculty of Medicine

Lund University

Lund 2026

ISBN 978-91-8021-853-5 (print)

ISBN 978-91-8021-853-5 (electronic)

Series title: Lund University, Faculty of Medicine Doctoral Dissertation Series 2026:55

ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University,
Lund, 2026



Media-Tryck is a Nordic Swan Ecolabel
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“Everyone who is born holds dual citizenship, in the kingdom of the well and in the kingdom of the sick. Although we all prefer to use the good passport, sooner or later each of us is obliged, at least for a spell, to identify ourselves as citizens of that other place”

– Susan Sontag, *Illness as Metaphor*

Table of Contents

Abbreviations	10
Declaration of AI use	12
Abstract	13
Popular Scientific Summary	14
Populärvetenskaplig sammanfattning	15
Papers Included in this Thesis.....	18
Papers Not Included in this Thesis.....	19
Introduction	20
Cancer	21
Hallmarks of Cancer.....	22
Brain Tumors.....	25
Glioblastoma	25
Clinical Manifestation and Prognosis	25
Treatment	26
Glioblastoma heterogeneity	26
Molecular Pathways in GBM.....	26
Glioma Cells with Stemness Properties	29
Glial Cells	32
Glial Development	32
Astrocyte Subtypes	33
Astrocyte Reactivity.....	34
Astrocytes in CNS pathologies	35
Astrocytes in the Context of Brain Tumors	36
The Tumor Microenvironment	39
The Blood Brain Barrier	39
Hypoxia.....	39
Hypoxia-Inducible Factors	40
Cellular Response to Hypoxia.....	41
Tumor Hypoxia	41
Stromal cell response to hypoxia	42

Radiation Induced Alterations of the Tumor Microenvironment	44
Tumor Cell Responses to Radiotherapy	44
Stromal Cell Responses to Radiotherapy	45
The Complement System	50
Complement in Cancer	51
The Present Investigation	55
Methodologies	55
Paper I: Coevolution of neoplastic and non-neoplastic reactive astrocyte states converges on mesenchymal-like and injury-response programs during murine glioblastoma progression and post-radiotherapy recurrence	58
Major findings	58
Paper II: Phenotypic Screening Identifies Flunarizine as an Inhibitor of Radiotherapy-Induced Astrocyte Reactivity with Therapeutic Potential in Glioblastoma	61
Major findings	61
Paper III: Hypoxia-Induced Complement Component 3 Promotes Aggressive Tumor Growth in the Glioblastoma Microenvironment	62
Major findings	62
Paper IV: Hypoxia-Induced Reactivity of Tumor-Associated Astrocytes Affects Glioma Cell Properties	64
Major findings	64
Summary and future perspectives	67
Acknowledgements	69
References	73

Abbreviations

AC	Astrocyte
ACR	Astrocyte reactive
AML	Acute Myeloid Leukemia
ATP	Adenosine triphosphate
BBB	Blood brain barrier
CAF	Cancer associated fibroblast
CCL	C-C motif chemokine ligand
CFB	Complement factor B
CNS	Central nervous system
CSC	Cancer stem cell
DAMP	Damage associated molecular patterns
DLK1	Delta-like non-canonical Notch Ligand 1
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Endothelial to mesenchymal transition
FGF2	Fibroblast growth factor 2
GBM	Glioblastoma
G-CIMP	CpG island methylator phenotype
GDNF	Glial cell derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GSC	Glioma stem cell
GSEA	Gene set enrichment analysis
Gy	Gray
HIF	Hypoxia inducible factor
hPDGFB	Human platelet derived growth factor B
HRE	Hypoxia response element

IDH	Isocitrate dehydrogenase
IL	Interleukin
INF	Interferon
IR	Irradiation
MES	Mesenchymal
MGMT	O6-methylguanine-DNA methyltransferase
NF1	Neurofibromatosis type 1
NK	Natural killer
NPC	Neural progenitor cell
NSC	Neural stem cell
NT	Non-telencephalon
OCT4	Octamer-binding transcription factor 4
OLIG2	Oligodendrocyte Transcription Factor 2
OPC	Oligodendrocyte progenitor cell
PCA	Principal component analysis
PD-1	Programmed cell death protein 1
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PHD	Prolyl hydroxylase domain
PD-L1	Programmed cell death-ligand 1
pVHL	Von Hippel Lindau tumor suppressor protein
PMT	Proneural to mesenchymal transition
PTEN	Phosphatase and tensin homolog
RCAS	Replication-competent retroviral vectors
RFP	Red fluorescent protein
RGC	Radial glial cell
ROS	Reactive oxygen species
SASP	Senescence-associated secretory phenotype
ScRNA-seq	Single cell RNA sequencing

SOX2	SRY-box transcription factor 2
SVZ	Subventricular zone
TCGA	The cancer genome atlas
TE	Telencephalon
TERT	Telomerase reverse transcriptase
TGF	Transforming growth factor
TME	Tumor microenvironment
TMZ	Temozolomide
TNF	Tumor necrosis factor
TP53	Tumor protein 53
TTFIELDS	Tumor treating fields
VEGF	Vascular endothelial growth factor
VIM	Vimentin
VZ	Ventricular zone
WHO	World health organization

Declaration of AI use

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Abstract

Glioblastoma represents the most aggressive primary brain tumor in adults. Despite intense treatment with surgery and chemoradiotherapy, treatment resistant tumors inevitably recur in close proximity to the primary lesion. Thus, underscoring the need for new therapeutic approaches. Glioblastomas are highly heterogeneous tumors, displaying various of molecular and phenotypic properties apart from consisting of a wide range of other cells, such as stromal and immune cells.

The aim of this thesis was to investigate how the non-malignant components of the tumor microenvironment contribute to tumor regrowth. The impact of radiation and hypoxia on non-tumoral cells remain poorly understood, even though these cells reside with tumor cells in the same areas and are exposed to the same treatment. Here, we used a murine GBM model to perform single cell RNA-sequencing and spatial transcriptomics, together with publicly available human data to get further insight to the changes in the tumor microenvironment. We performed *in vitro* functional studies using primary stromal and tumor cells to evaluate possible tumor-driving mechanisms, and finally, confirmed some of these findings in pre-clinical settings. In paper I, we showed that radiotherapy caused both tumor and stromal cells to converge toward mesenchymal and reactive-astrocyte-like phenotypes. This AC/MES shift was tightly linked to high injury-response programs, fitting well with several conceptual frameworks that OPC/NPC drive towards AC/MES-states and a developmental-injury continuum. This transition appeared to be driven in part by highly reactive astrocytes with strong ECM-remodeling interactions with tumor cells. In paper II, we explored whether radiotherapy-induced stromal reactivity could be pharmacologically reversed using a drug-repurposing approach. We identified a compound that modulated the reactive extracellular matrix after radiotherapy resulting in reduced glial-scar formation. In paper III we focused on hypoxic niches and innate immune reactions. We showed that hypoxia and complement activation converge to promote tumor progression by immunosuppression. Paper IV showed that hypoxia induces a pronounced reactive shift of astrocytes, and that their altered ECM expression promotes GBM survival, aggressiveness and stemness.

Overall, this thesis provided further insights into the complex glioblastoma microenvironment, where both radiotherapy and hypoxia increase stromal-cell mediated tumor progression and radioresistance, which provide insights to possible feature target treatments.

Popular Scientific Summary

Glioblastoma is the most common and deadliest brain cancer. Despite intense treatment with surgery, radiotherapy and chemotherapy, tumors almost always come back, often in close relation to the site of the primary tumor. For decades, researchers have focused on understanding the properties of tumor cells, and how they survive and resist therapies. However, there is growing evidence that other components other than tumor cells themselves can play crucial roles in tumor cell progression and survival. Tumors are composed of several cell types apart from the cancer cells. They include stromal cells, such as fibroblast, endothelial cells and pericytes that build the structural framework and provide oxygenation of tumors. They also contain a repertoire of immune cells, including tumor-associated macrophages, B cells, T cells, NK cells and neutrophils. Additionally, the whole environment is filled with non-cellular components, such as extracellular matrix, signaling molecules and physiological factors, for instance areas of low oxygen and borders exposed to remaining tissue. All these components and compartments together create an interactive ecosystem within tumors, called the tumor microenvironment (TME). Brain tumors differ from other solid tumors by the additional layer of the blood brain barrier (BBB), that restricts immune cell infiltration, and adds to the complexity of cancer treatment, as many drugs cannot pass this physical barrier. The brain TME also includes specialized brain-resident cells, such as microglia, astrocytes, oligodendrocytes and neurons, making this ecosystem particularly context-dependent. Research has shown that many of these non-tumor cells get directed to fuel tumor grow and spread, not only through stromal-tumor interaction, but also through interactions with each other.

Glioblastomas are characterized by certain physical compartments. They are fast growing tumors, and rich in areas with low oxygen, called hypoxic regions. It is known that tumor cells in these areas respond worse to treatment, both due to the lack of oxygen, but also the inaccessibility of drug delivery. The different compositions of the microenvironment in tumors have been linked to variations in immune cell, stromal, and matrix composition.

These diverse physical compartments all represent a complex environment that is contributing to tumor progression in their own separate ways. Apart from this, the tumor cells themselves exhibit a broad diversity within the same tumor. They are derived from glial cells and can exhibit a spectrum of characteristics from these cell types, such as oligodendrocyte progenitor-like, proneural-like, astrocyte-like or mesenchymal-like signatures. Tumor cell can therefore display large plasticity and come in several flavors, and they are also able to convert between these states depending on the environmental pressure. There is limited knowledge on how tumor cells convert between states, and how the microenvironment influences this behavior. But also, there is a general lack of understanding of how the non-tumor

cells behave in response to treatment. Increased understanding of the above could potentially open up for future treatments that do not only target tumor cells themselves, but also the remaining tissue that supports the tumor in growing back.

This thesis investigates how radiotherapy and hypoxia reshapes the glioblastoma microenvironment with a particular focus on astrocytes, which are a star-shaped support cell in the brain that normally protects and provide nutrients to neurons. Here, across four separate interconnected studies, we have characterized how astrocytes respond to stress, such as radiation or lack of oxygen, by inducing a reactive state with altered behavior that helps the tumor become more aggressive. We found that both tumor cells and stressed astrocytes begin to behave in surprisingly similar ways during disease progression, by activating gene programs that are normally seen in injury and inflammation. This blurs the line between what is considered tumor and non-tumor compartments and highlights that astrocyte features can actively drive cancer progression, particularly after radiotherapy. In response to damage, reactive astrocytes typically start producing proteins that rebuild the matrix to isolate the area of brain injury from remaining healthy tissue, called glial scar. Using a drug-screening approach, we found that an already approved anti-migraine medicine could block harmful astrocyte activation, and limit the scar formation after radiation, which leads to slower tumor growth and increased survival. Similarly to radiation, low oxygen also modulated the functions of astrocytes to support tumor stemness and resistance to treatment. Surprisingly, we found that areas in the tumors with lower oxygen had local expression of part of the innate immune system, called the complement system. Here, it was not just astrocytes, but many stromal cell types, including tumor cells themselves that increased expression of the central component of the cascade, called C3. Therapeutic blockage of C3a/C3aR-signaling lead to a changed immune microenvironment and improved the survival of mice. Interestingly, this links back to the injury-response profile seen with tumor progression, as parts of the complement system and inflammatory signaling promote both astrocyte reactivity, and injury-response programs of glioblastoma cells. Overall, the work of this thesis shows that glioblastoma is not just a disease of cancer cells but is a dynamic ecosystem where non-cancerous cells can be reprogrammed and fuel malignancy, partially through injury and inflammatory pathways.

Populärvetenskaplig sammanfattning

Glioblastom är den mest vanliga och dödligaste formen av hjärntumör. Även om den behandlas intensivt med kirurgi, strålning och cellgifter återkommer tumören, ofta i samma område som primärtumören. Forskning har länge fokuserat på hur egenskaper av tumörcellerna bidrar till att de kan överleva och bli resistenta mot behandling. Det finns dock växande bevis för att andra komponenter än

tumörcellerna själva kan spela avgörande roller i tumörernas progression och överlevnad. Tumörer består av flera celltyper förutom cancercellerna själva. De inkluderar bland annat stromala celler, så som fibroblaster, endotelceller och pericyter som bygger upp de strukturella ramarna och förser tumören med syre och näring. De innehåller också en bred repertoar av immunceller, så som tumörassocierade makrofager, B-celler, T-celler, NK-celler och neutrofiler. Dessutom är hela miljön fylld med icke-cellulära komponenter, så som extracellulär matrix, signalmolekyler och fysiologiska särskilda områden med låg syrehalt eller där tumören avgränsas till frisk vävnad. Alla dessa komponenter och områden skapar tillsammans ett interaktivt ekosystem inom tumörer, kallat tumörmikromiljön. Hjärntumörer skiljer sig från andra solida tumörer genom det extra lagret av blod-hjärnbarriären, som begränsar immuncellsinfiltration och ökar komplexiteten för cancerbehandling eftersom många läkemedel inte kan passera denna fysiska barriär. Hjärntumörens mikromiljö inkluderar även specialiserade hjärnceller, såsom mikroglia, astrocyter, oligodendrocyter och neuroner, vilket gör detta ekosystem särskilt kontextberoende. Forskning har visat att många av dessa icke-tumörceller styrs att driva tumörtillväxt och spridning, inte bara genom att interaktionen mellan tumör och stroma, utan även också genom interaktioner med varandra.

Glioblastom kännetecknas av vissa karaktärsdrag. De är snabbväxande tumörer och rika på områden med låg syrehalt, kallade hypoxiska regioner. Det är känt att tumörceller i dessa områden svarar sämre på behandling, både på grund av syrebrist men också på grund av otillgängligheten av läkemedelsleverans. Dessutom skapar tumörgränsen, som är det område som avskiljer frisk från sjuk vävnad, en annan miljö från resterande av tumören. Dessa olika sammansättningar av mikromiljön i tumören har kopplats till variationer i immuncells- stromal- och matrixsammansättning, men också egenskaper hos tumörcellerna. Dessa olika fysiska avdelningar representerar komplexa miljöer som bidrar till tumörprogression på sina egna sätt. Bortsett från detta, uppvisar tumörcellerna själva en bred mångfald. De härstammar från gliaceller, och kan uppvisa ett spektrum av egenskaper från dessa, med signaturer som överlappar med oligodendrocyter, neurala, astrocyt eller mesenkymala program. Cancerceller uppvisar därför stor diversitet inom tumörer, och kommer i många olika varianter. De har även visats att de besitter en stor plasticitet, och kan konvertera mellan dessa tillstånd beroende på omständigheter. Det finns dock begränsad kunskap om hur tumörceller konverterar mellan tillstånd, och hur mikromiljön samt behandlingar påverkar dessa beteenden. Men det finns också en allmän brist på förståelse för hur icke-tumörceller beter sig i relation till cancerbehandling. En ökad förståelse inom ovanstående, skulle potentiellt kunna öppna framtida behandlingar som inte bara riktar sig mot tumörceller själva, utan även mot den återstående vävnaden som hjälper till att driva tumörens återtillväxt.

Denna avhandling undersöker hur strålbehandling och hypoxiska områden omformar glioblastoms mikromiljö, med särskilt fokus på astrocyter. Astrocyter är en vanligt förekommande stjärnliknande stromacell, som normalt sett skyddar och förser neuroner med näring. I våra studier, har vi karakteriserat hur astrocyter skiljer sig från frisk hjärna och närvaro av tumör, samt hur de reagerar på stress såsom strålning eller syrebrist och inducerar ett reaktivt tillstånd med förändrat beteende som hjälper tumören att bli mer aggressiv. Vi fann att både tumörceller och stressade astrocyter börjar bete sig på ett förvånansvärt liknande sätt under sjukdomsprogression, genom att aktivera genprogram som normalt ses vid skada och inflammation. Detta suddar ut gränserna mellan vad som anses vara tumör- och icke-tumörkomponenter och belyser att astrocytfunktioner aktivt kan driva cancerprogression, särskilt efter strålbehandling. När det sker en hjärnskada, börjar astrocyter förändra sina egenskaper, bli reaktiva, och producera proteiner som återuppbygger extracellulär matrix i en ärrliknande procedur för att isolera området i hjärnan där skadan har skett från resterande frisk vävnad. En process som kallas glios. Med hjälp av en ny metod som vi använde, kunde vi screena flera tusen läkemedel, och fann att ett redan godkänt läkemedel mot migrän kunde blockera skadlig astrocytaktivering och begränsa ärrbildning efter strålning, vilket resulterade i långsammare tumörtillväxt och ökad överlevnad. I likhet med strålning, modulerar låg syrehalt också astrocyternas funktioner för att stödja tumörfrämjande egenskaper. Intressant nog, hittade vi en stark koppling mellan hypoxiska zoner i tumören och lokalt uttryck av en del av det medfödda immunsystemet, kallat komplementsystemet. Det var inte bara astrocyter, utan även många andra stromala celltyper, samt tumörcellerna själva som ökade uttrycket av den centrala komponenten i kaskaden, kallad C3. När vi blockerade delar av denna signalering, genom C3a/C3aR-blockering, förändrades mikromiljön i tumörerna och vi såg förbättrad överlevnad i djurstudier. Detta återkopplar till skaderesponsprofilen som ses vid tumörprogression, eftersom delar av komplementsystemet och inflammatorisk signalering främjar både astrocytaktivitet och ett skaderesponsprogram hos glioblastom celler själva. Sammantaget, bygger denna avhandling vidare på konceptet att glioblastom inte bara är en sjukdom av cancerceller, utan ett dynamisk ekosystem där icke-cancerösa celler kan omprogrammeras och ge näring åt tumören, delvis genom skade- och inflammatoriska gensvar.

Papers Included in this Thesis

Paper I

Coevolution of neoplastic and non-neoplastic reactive astrocyte states converges on mesenchymal-like and injury-response programs during murine glioblastoma progression and post-radiotherapy recurrence.

Lindgren, D. *, **Rosberg, R. ***, Smolag, K. I., Manou, D., Sjölund, J., Braun, S., Phung, B., Harbst, K., Johansson, E., Jeannot, P., Ceberg, C., Jönsson, G. B., Pietras, K. & Pietras, A.

*These authors contributed equally.

Preprint BioRxiv 2025; <https://doi.org/10.64898/2025.12.18.695184>

Paper II

Phenotypic Screening Identifies Flunarizine as an Inhibitor of Radiotherapy-Induced Astrocyte Reactivity with Therapeutic Potential in Glioblastoma

Jeannot, P., **Rosberg, R.**, Lindgren, D., Dawson, J. C., Pracucci, E., Börjesson-Freitag, C., Martinez, J., Pantazopoulou, V., Malmberg, M., Smolag, K. I., Manou, D., Elliot, R. J.R., Ceberg, C., Berg, T. J., Ahlenius, H., Carragher, N. O. & Pietras, A.

Submitted Manuscript

Preprint BioRxiv 2025; <https://doi.org/10.1101/2025.07.12.664538>

Paper III

Hypoxia-Induced Complement Component 3 Promotes Aggressive Tumor Growth in the Glioblastoma Microenvironment

Rosberg, R., Smolag, K. I., Sjölund, J., Johansson, E., Bergelin, C., Wahldén, J., Pantazopoulou, V., Ceberg, C., Pietras, K., Blom, A. M., & Pietras, A.

JCI insight, 2024; 9(19). <https://doi.org/10.3390/cells10030613>

Paper IV

Hypoxia-Induced Reactivity of Tumor-Associated Astrocytes Affects Glioma Cell Properties

Pantazopoulou, V., Jeannot, P., **Rosberg, R.**, Berg, T. J. & Pietras, A.

Cells 2021; 10(3). <https://doi.org/10.3390/cells10030613>

Papers Not Included in this Thesis

Pericytes Orchestrate a Tumor-Restraining Microenvironment in Glioblastoma

Braun, S., Bolivar, P., Oudenaarden, C., Sjölund, J., Bocci, M., Harbst, K., Talkhoncheh, M. S., Phung, B., Cordero, E., **Rosberg, R.**, Johansson, E., Jönsson, G. B., Pietras, A., & Pietras, K.

Nature communications, 2025; 16(1)

<https://doi.org/10.1038/s41467-025-66985-1>

Complement Factor H Is an ICOS Ligand Modulating Tregs in the Glioma Microenvironment

Smolag, K. I., Olszowka, J., **Rosberg, R.**, Johansson, E., Marinko, E., Leandersson, K., O'Connell, D. J., Governa, V., Tuysuz, E. C., Belting, M., Pietras, A., Martin, M., & Blom, A. M.

Cancer immunology research, 2025; 13(1)

<https://doi.org/10.1158/2326-6066.CIR-23-1092>

Tumor Suppressor Role of the Complement Inhibitor CSMD1 and its Role in TNF-Induced Neuroinflammation in Gliomas

Tuysuz, E. C., Mourati, E., **Rosberg, R.**, Moskal, A., Gialeli, C., Johansson, E., Governa, V., Belting, M., Pietras, A., & Blom, A. M.

Journal of experimental & clinical cancer research, 2025; 43(1)

<https://doi.org/10.1186/s13046-024-03019-6>

Introduction

The next few minutes were the longest of my life. A bit like that last minute when you're standing and waiting for the washing machine to finish. I paced back and forth in the lab, trying to continue my experiment, but I couldn't focus. A quiet panic built up inside me – a certainty that something was very wrong. Just a few minutes later, my phone rang.

“We received an alarm regarding your blood tests from this morning, and we need you to go to the nearest emergency department immediately”, the nurse said. I decided to ask directly what this meant and why they wanted me to go. “We want to rule out malignancy”.

The room was dark and empty when I arrived. In the middle stood a narrow bed with an office chair beside it. The walls were bare except for a small round window looking out into the corridor. The yellow light from the headlights in the corridor displayed the silhouettes of bodies in motion. But in the room, everything stood still. The staff wore isolation masks every time they entered. She sat down on the edge of the bed and placed her hand on my thigh just above my knee. She looked at me with a meaningful, slightly sad expression, apologizing that I had ended up there. She promised that I would walk out of the hospital again. I didn't really understand what she meant – of course I would leave this hospital! But little did I know that a small paper cut could have devastating consequences. The large bag of yellow fluid began to flow into my arm, while the life I had known slowly flowed out that round window in the corridor door.

Every day around 55 000 people get a cancer diagnosis, leading up to approximately 20 million new cases globally per year¹. Cancer is only one word, but changes everything of an individual's life from the moment it is spoken out loud. It is common for patients to experience helplessness and loss of control and identity². Due to advances in cancer research and treatment, today 70% of patients survive 5-years after their diagnosis³. However, the yearly cancer incidences are rising, with an estimation to 35 million cases globally in 2050¹. Concerningly, there is a rapid increase in early onset cancers, affecting young adults under the age of 40⁴. This means that a growing number of individuals, especially those diagnosed at younger

ages, are surviving longer after cancer. This underscores the increasing need to develop more targeted therapies, and understanding long-term side effects and consequences, such as elevated risks of secondary malignancies, cognitive deficits, chronic fatigue, cardiovascular complications, psychological distress, and hormonal dysfunction or infertility⁵.

Cancer is a disease that has long confused scientists. Today we know that some cancer risks are hereditary, for example, inherited mutations in the *BRCA1* and *BRCA2* genes are major drivers in breast and ovarian cancer, whereas most cancers arise through acquired mutations during the lifetime⁶. Our cells can accumulate genetic damage from various sources, including chemicals, smoking, UV, and ionizing radiation. Meanwhile, certain cancers such as cervical, and head and neck cancers, can be caused by viruses such as Human Papilloma Virus (HPV)⁷. We know today that there are more than 200 different types of cancer.

The treatments available for combating cancer primarily aim to target malignant cells while minimizing damage to healthy tissue. Since cancer cells are transformed from our own cells, it is inherently challenging to target cancerous cells specifically. Traditional therapies rely on cytotoxic agents and radiotherapy that kill or target rapidly dividing cells. While these agents effectively impair cancer cells, they also harm normal tissues with high turnover rates. Treatment therefore requires balancing of the dose needed to eradicate tumor cells, with the lowest possible toxicity on healthy tissue. More sophisticated therapeutic strategies have been developed to better discriminate between malignant and non-malignant cells. For example, immunotherapies, such as Programmed Cell Death Protein 1 and Programmed Cell Death-Ligand 1 (PD-1/PD-L1) checkpoint inhibitors were awarded the Nobel Prize in 2018, and have shown remarkable effects in melanoma, and are currently being evaluated in several other cancer types⁸. Another strategy is the use of differentiation therapy, which aims to force malignant cells to mature into less aggressive cells which can be killed more easily⁹. As cancer cells often exist in immature, developmentally arrested states, with high plasticity and uncontrolled growth, agents (such as retinoic acid) force them to cellular differentiation and can drive them to maturation and ultimately cell death.

Despite our increased knowledge and advances in therapies, the prognosis for certain cancers remains poor, among them is the most common type of brain tumor, called glioblastoma, that this thesis work is focused on.

Cancer

Cancer is a complex disease characterized by uncontrolled cell growth of abnormal cells. Douglas Hanahan and Robert A. Weinberg elegantly summarized the hallmark traits of cancers when publishing “The hallmarks of Cancer”¹⁰⁻¹³. Overall,

based on the last decades of research, there are currently 14 known traits to describe common characteristics of cancer cells.

Hallmarks of Cancer

Self-renewal and endless divisions

Tumor cells have a unique capability of self-renewal. While normal cells are dependent on external signals to initiate growth, tumor cells sustain proliferative signaling by intrinsically upregulating autocrine production of growth factors, or increasing the cell surface receptors responsible for proliferative signaling. Many tumors harbor mutations in genes monitoring cellular growth. These allow for excessive downstream proliferative signaling even in the absence of external stimuli¹⁰. However, tumors also often modulate neighboring cells within the TME to produce growth factors, which in turn, support tumor cell growth through paracrine signaling. Opposite to proliferation, tumor cells also possess traits to evade growth suppressors that regulate cell division in healthy cells, called tumor suppressor genes. One of the most frequently mutated and studied tumor suppressor gene is tumor protein p53 (TP53)¹⁴. It acts as a transcription factor, that becomes activated in response to cellular stress and governs genomic aberrations. In case of deoxyribonucleic acid (DNA) damage, it either assists to repair the injury, or if beyond rescue, it induces cellular apoptosis. In this way, TP53 helps maintain tissue homeostasis by eliminating damaged cells in a controlled manner. Loss of TP53 function is often seen in cancers, resulting in disrupted apoptotic pathways leading to cancer cell survival and their ability to resist cell death. Other common ways for tumors to achieve this, is to modulate cell death signaling by upregulating anti-apoptotic factors, such as Bcl-2 and Mcl-1 or downregulating pro-apoptotic factors¹⁵. Apart from this, tumor cells can divide infinitely, through replicative immortality. This is achieved through bypassing the normal cellular aging. Healthy cells normally have a limited number of cell cycles through their life span, which is why we age. Each time a cell divides, its genome becomes slightly shorter, this is why, chromosomal ends are covered with protective segments of repeated DNA in the chromosomal ends, called telomeres. Tumor cells upregulate the enzyme responsible for maintaining telomeres, thereby ensuring their immortality¹⁶. Other intrinsic factors that characterize tumors cells are their genomic instability and mutations, and nonmutational epigenetic reprogramming. The first of these is based on alterations of the cancer cells DNA sequence, including DNA breaks, copy-number changes, point mutations and chromosomal rearrangements¹⁷. Alterations of the cancer cell genome can appear early in the tumorigenesis, or cell of origin, and is then called clonal, whereas if mutations appear later in the process, they are considered subclones, usually a result of genomic instability or adaption to stress with survival advantages. Non-mutational epigenetic programming refers to

changes in gene expression without alterations to the underlying DNA sequence, that are regulated by DNA methylation, histone modifications and chromatin remodeling¹⁸. Epigenetic shifts of tumor cells enable them to rapidly adjust transcriptional programs, promoting plasticity, differentiation and de-differentiation when needed.

Together, these characteristics define the characteristics hallmarks of *sustained proliferative signaling, evading growth suppressors, enabling replicative immortality, resisting cell death, nonmutational epigenetic reprogramming and genome instability and mutation* of cancer cells.

Cellular plasticity and adaptation

Tumor development and progression rely on a complex interplay of several biological processes that enables malignant cells to survive, expand and disseminate. This includes accessing vasculature, from which cancer cells secure a continuous supply of oxygen and nutrients, maintaining sustained growth. As tumors evolve, they activate programs of invasion and metastasis, reforming the cell-cell and cell-matrix interactions to allow migration into the circulation and over tissue boundaries, a process called endothelial to mesenchymal transition (EMT). Phenotypic plasticity enables cancer cells to transition between distinct functional states, contributing to a diverse tumor heterogeneity, therapeutic resistance, metastasis and survival in nutrient-limited niches, such as hypoxia. Integral to this phenotypic adaptability, is deregulation of cellular metabolism, in which malignant cells shift their metabolic pathways to meet their surroundings. In response to damage, or cellular stress, some tumor cells adapt a senescent state, recognized by their arrest in cell cycle, but remaining metabolically active with a senescence-associated secretory phenotype (SASP), which can protect neighboring tumor cells from treatment-induced stress¹⁹. All of the above are examples of cancer hallmarks including of *inducing or accessing vasculature, unlocking phenotypic plasticity, activating invasion and metastatic programs, deregulating cellular metabolism and becoming senescent*.

Inflammation and immune regulation

Tumor-promoting inflammation describes the importance of how secreted cytokines, growth factors and reactive molecules from stromal and immune cells of the TME contribute to tumor progression by creating conditions that support tumor growth and tissue remodeling. Simultaneously, many tumor cells must avoid immune destruction by expressing markers that dampens the response of immune cells, or downregulating markers that call immune attention. The immune system can therefore play dual roles in tumor growth. Finally, the latest addition, controversially discuss the presence of polymorphic microbes, such as resident tumor bacteria or tissue microbes to be considered a hallmark of cancer. These are

the hallmarks of *tumor-promoting inflammation*, *avoiding immune destruction* and *tumor promotion by interacting with polymorphic microbes*.

Together, the fourteen hallmarks of cancer describe how malignant cells acquire capacities of endless self-renewal, adapt to their surroundings under pressure, and escape intrinsic and extrinsic-mediated cell death. Collectively, these drive tumor initiation, progression and therapeutic resistance.

Brain Tumors

Brain tumors are classified based on their cellular origin and tumor characterization, including immunohistochemistry and histology. Gliomas are derived from glial origin, and are the most common type of primary brain tumors, representing 80% of malignant cases. Adult diffuse gliomas are divided into astrocytoma isocitrate dehydrogenase (IDH)-mutant, oligodendroglioma IDH-mutant and 1p/19q-codeletion, and glioblastoma IDH-wildtype according to the latest update from World Health Organization (WHO)^{20,21}. Previously, in the 2016 WHO classification, IDH-mutant diffuse astrocytic gliomas were categorized into diffuse astrocytoma, anaplastic astrocytoma and glioblastoma²². Glioblastomas (GBMs) were then classified based on their IDH status, with absence or presence of mutations in either IDH1 or IDH2. In contrast, the current classification considers only IDH-wildtype to be GBM. Apart from being IDH-wildtype, GBMs are also characterized of having pseudopalisading necrosis, high mitotic activity and microvascular proliferation with either one or more of the following three genetic parameters; epidermal growth factor receptor (EGFR) gene amplification, Telomerase Reverse Transcriptase (TERT) promoter mutation, chromosome 7 gain and loss of chromosome 10.

Glioblastoma

Clinical Manifestation and Prognosis

GBM is most commonly diagnosed in adults of 40-70 years, with a median age at diagnosis of 64²³. The incidence is higher in men compared to females. Few risk factors are known about the development of GBM²⁴. Prior exposure to radiotherapy has been shown to increase the risk. Beyond this, there is no evidence that lifestyle factors such as alcohol consumption or cigarette smoking, both known factors contributing to many cancers, are associated with GBM risk. Clinical manifestations typically include headaches, cognitive impairments, nausea, seizures, motor deficit and visual changes²⁵. The prognosis of GBM remains poor, and fewer than 5% of patients survive five years after diagnosis, with a median survival of approximately 15 months^{26,27}.

Treatment

Despite advances in GBM research, the treatment options for patients remains limited. Standard of care therapy includes maximal safe surgical resection, followed by temozolomide (TMZ) chemotherapy and fractionated radiotherapy with a total of 60 Gy over 30 days distribution^{28,29}. Due to the infiltrative growth patterns of GBM, complete removal of all malignancy is difficult to achieve, and residual cancer cells often remain. The extent of tumor removal is one of the few treatment-related prognostic factors of GBM, where patients that receive complete surgical removal survive longer compared to patients who do not achieve this³⁰. Additionally, patient survival was improved by the introduction of TMZ and radiotherapy³¹⁻³³. Nevertheless, O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation is a critical prognostic marker for the efficiency of TMZ treatment, as the MGMT protein repairs DNA damage caused by alkylating therapies, such as TMZ^{34,35}. The clinical usefulness of MGMT status remains limited, primarily due to the absence of effective therapies for patients with GBM harboring an unmethylated MGMT promoter³⁶. In addition, patients can be treated using tumor treating fields (TTFields), which is a localized, anti-cancer technique using a non-invasive, wearable device that generates alternating electric fields across the tumor site. The proposed mechanism of action includes impaired cellular proliferation, by disrupting mitosis, DNA replication and cell repair^{37,38}. Despite these intense treatment efforts, tumors inevitably recur in close proximity, usually within 2 cm to the primary tumor site^{39,40}. Currently, there is no universal standard of care recommendations for recurrent GBM treatment⁴¹, and treatment option remains limited as recurrent tumors often exhibit resistance to primary treatments. In less than 50% of cases, patients are suitable for re-operation, which has been linked to longer survival⁴²⁻⁴⁴. Apart from this, patients therefore rely on ongoing clinical trials or development of new therapies. Currently, ongoing clinical trials include anti-vascular endothelial growth factor (VEGF) treatments, MET-inhibitors, additional alkylating agents, and immunotherapy⁴⁵⁻⁴⁷. Unfortunately, these have yielded moderate or no promising results.

Glioblastoma heterogeneity

Molecular Pathways in GBM

Molecular signatures in GBM were first introduced in studies from Phillips, Verhaak, and Wang *et al.* based on gene-expression profiles⁴⁸⁻⁵⁰. Using bulk RNA sequencing from The Cancer Genome Atlas (TCGA), Verhaak *et al.* showed in 2010 that GBM could be divided into four different subgroups; proneural, neural, classical and mesenchymal based on their transcriptional profile, although the neural

subtype later was shown to be a contamination of normal cells⁵⁰. The proneural GBM subtype was characterized by PDGFRA amplification and loss-of-function of TP53⁵¹. Historically, this subtype was divided into two subgroups, gliomas with CpG island methylator phenotype (G-CIMP), marked with hypermethylated DNA and typically associated with IDH-mutations, and a non-G-CIMP proneural subgroup⁵². IDH-mutations were considered a favorable prognostic outcome for patients, with longer survival⁵³. However, according to the current classifications, G-CIMP and IDH-mutant proneural gliomas are no longer classified as GBMs but are instead designated as IDH-mutant astrocytoma, grade IV²¹. In contrast, the classical GBM subtype was mostly associated with EGFR activation and Phosphatase and Tensin homolog (PTEN) loss, driven by chromosome 7 amplification and chromosome 10 loss, genetic alterations present in nearly all classical GBMs⁵¹. The mesenchymal subtype was defined by frequent loss of PTEN and NF1. Individuals harboring NF1 mutations generally have an increased predisposition to tumor formation due to overactive RAS/MAPK pathway signaling, and NF1 loss is one of the few inherited genetic risk factors for gliomas aside from TP53 and PTEN mutations⁴⁹. Mesenchymal GBMs exhibited elevated expression of mesenchymal and astrocytic markers such as Vimentin (VIM), CD44 and EMT-associated transcriptional factors including TWIST1, Snail and Snai2. Compared with the other subtypes, mesenchymal tumors show greater immune cell infiltration and a higher degree of tumor necrosis. Overall, most GBMs harbors abnormalities in all the above-mentioned pathways (PTEN, TP53 and receptor tyrosine kinase pathways), suggesting their core involvement in GBM pathogenesis. However, despite their transcriptional differences, there was no clinically significant outcome between the subgroups of GBMs. Which could be explained by the fact that GBM tumors display a remarkable cellular heterogeneity and extensive transcriptional diversity, inherently making molecular classification difficult. While the bulk signature reflected a predominant molecular pathway, multiple subtypes clearly coexist within individual tumors^{51,54-60}. The introduction of single-cell RNA sequencing (scRNA-seq) has significantly deepened our understanding of the tumor complexity and offers a more effective approach for dissecting tumor heterogeneity⁶¹. Unlike bulk sequencing, scRNA-seq makes it possible to investigate the transcriptional states of individual tumor and stromal cells rather than only their shared transcriptomic profiles.

Plasticity

One of the earliest studies using scRNA-seq, published by Patel *et al.* profiled 430 cells derived from primary GBM tumors. This study revealed profound transcriptional heterogeneity among malignant cells, despite their shared common chromosomal abnormalities. The transcriptional variation was linked to hypoxia-related pathways, immune-response programs, proliferation-associated genes and oncogenic signaling⁶². The overall transcriptional signature could be traced back to

the original TCGA subtypes, including proneural, classical and mesenchymal signatures. However, on single-cellular level, they showed that all subtype-associated programs co-existed within individual tumors.

Furthermore, as the sequencing technique evolved, larger and more comprehensive datasets could be generated. Neftel *et al* introduced four cellular states of GBM cells: neural-progenitor-like (NPC-like), oligodendrocyte-progenitor-like (OPC-like), astrocyte-like (AC-like) and mesenchymal-like (MES-like)⁶³. All four states are found in GBM tumors, but their relative abundance varies and are linked to their genetic alteration, such as amplification of Cyclin-Dependent Kinase 4 (CDK4), PDGFRA, EGFR and NF1 loss, which preferentially drive the proneural, classical and mesenchymal subtypes, respectively. Using barcoding strategies, Neftel *et al.* also demonstrated that GBM cells exhibit substantial plasticity between these states and that individual clones from each state were able to recapitulate the full spectrum of cellular states, indicating that these programs are dynamic rather than terminally fixed. Such plasticity and heterogeneity may explain the treatment resistance and tumor recurrence in GBM, as limited differences between the composition of primary and recurrent brain tumors have been observed^{64,65}.

Spatial and temporal sampling has further highlighted that GBM cell states, as well as the TME, are strongly influenced by location and disease progression. For instance, studies analyzing region-specific biopsies have shown clear distinctions between tumor-core and tumor-border cells^{66,67}. Although our understanding of GBM phenotypes and cellular states has advanced, the dynamics of their relative abundance in association with tumor recurrence are less defined. Although, several separate studies have reported a PMT transition in response to radiotherapy or GBM recurrence^{48,68-72}, results are conflicting since some studies report no difference^{65,69}. In the reported cases, PMT is associated with tumor progression and therapy resistance via upregulation of mesenchymal markers (CD44, STAT3, CEBP, and Vimentin)^{48,72-75}. Additionally, recent studies from Garcia-Diaz *et al.* demonstrate that tumor cells exposed to the microenvironment in tumor margins reacquire properties of AC-like cells, and exhibit more resistance to TMZ⁶⁷. A recent publication from Zheng *et al.* using multiregional GBM samples revealed that transcriptional programs associated with tumor hypoxia, as well as tumor cells with AC-like signatures, correlate with poorer prognosis⁷⁶. Interestingly, AC-like tumor cells were linked to worse outcomes only when they exhibited increased spatial connectivity with one another, and not when interacting with other subtypes such as MES-, NPC-, or OPC-like cells. Moreover, the overall proportion of AC-like cells alone did not predict patient outcome. These findings suggest that the spatial organization of for instance, AC-like tumor cells, may play a more critical role in prognosis rather than their abundance. Another publication by Sojka *et al.* compared GBM cells with the developing trajectory of astrocytes, showing that AC-like tumor cells deviate from normal maturation and remain in an early developmental stage, a feature present in GBM but not IDH-mutant gliomas⁷⁷.

Hybrid and emerging cellular states

Apart from the glial and neuronal like comparisons, glioma cells also adopt a wide range of cellular programs. Early studies from Nefitel *et al.* showed presence of intermediate hybrid cellular states existing between NPC-, OPC, MES- and AC-like tumor cells. Since then, the plasticity of tumor cells has also been demonstrated by Richards *et al.* over a transcriptional continuum spanning from developmental, neuronal-like programs to injury-response signatures, that may be a driver of tumor recurrence⁷⁸. Interestingly, another study found that early tumorigenesis switches on injury-programs, that drive tumor progression mediated through the environment⁷⁹. Additionally, a reactive-hypoxia program, was shown to overlap with the injury response programs from Richards *et al.*, and mesenchymal subtypes from Verhaak and Nefitel *et al.* where areas of reactive-hypoxia were associated with accumulation of copy number alterations⁷³. Introduction of these injury response profiles may be a direct effect of how GBM injures the brain as it expands. A growing evidence shows that these injury-associated genes show a significant overlap with reactive astrocyte signatures^{78,80}, and the existence of a “MES-AC-hybrid” state⁸¹. These reactive AC-like states show overlap with MES signatures widely described in the literature, and could suggest that MES-like states comprise of cells that contain dormant reactivity triggered by microenvironmental stressors. While previous work has demonstrated that injury-response programs are engaged at the onset of tumorigenesis, it is still relatively unexplored how the injury-associated MES and reactive astrocyte (ACR) axes evolve during therapeutic stress. Exploring whether therapy further drives these programs, and whether this promotes resistance could reveal important therapeutic vulnerabilities if these programs could be neutralized or switched off.

Glioma Cells with Stemness Properties

The modern cancer stem cell (CSC) model in GBM originates from leukemia studies showing that only a rare subset of malignant cells sustain disease. In the 1990s, a small subset of acute myeloid leukemia (AML) cells capable of regenerating leukemia in immunodeficient mice was identified, demonstrating capacities of self-renewal and long-term repopulation^{82,83}. This work established the leukemia stem cell model and explained why therapies targeting rapidly dividing cells often fail, leading to relapse.

Building on leukemia studies, the CSC model was extended to solid tumors like GBM. Early work focused on identifying GBM cells with stem-like traits, such as tumor-initiation, self-renewal and therapy resistant clones. Identification of glioma stem cells (GSCs) was broadly based on expression markers such as CD133, CD44,

Octamer-binding transcription factor 4 (OCT4), SRY-box transcription factor 2 (SOX2) and Nestin, exhibiting a general stem-like signature found in many cancers⁸⁴⁻⁸⁹. However, studies showed that CSCs in GBM rather seem to display a dynamic spectrum of stem-like states, rather than a fixed cell type and therefore problematize the usage of single markers to study stem cells⁹⁰. For instance, the early scRNA-seq studies by Patel *et al.* provided the first strong evidence for multi-lineage cancer stem cells as they observed a continuum of stemness related states, indicating widespread partial stemness⁶². Furthermore, work from Dirkse *et al.* suggested that stem-like properties may extend across multiple cellular states in GBM, rather than being confined to any single subpopulation. This was based on their functional studies showing that isolated cells from all different malignant subpopulations possess properties of self-renewal and multipotency, and were able to reconstruct the original intra-tumoral heterogeneity when re-implanted *in vivo*⁹¹. Combinational targeting of heterogenous glioma cells with stemness features may therefore be a promising avenue⁹². Together, the above reinforces the concept of GBM showing cellular plasticity as described in the previous chapter, and that stemness properties are dynamic and reversible across all tumor cell populations, which are highly influenced by certain microenvironmental niche factors.

Extrinsic cues in the GBM microenvironment play a key role in driving and maintaining stemness. Glioma cells with increased stemness are generally associated with distinct GBM tumor niches, including perivascular, invasive and perinecrotic regions⁹³. For instance, cells with stem-like features, such as proneural-like GSCs, are enriched in perivascular regions, and mesenchymal-like cells in perinecrotic areas^{94,95}. Extrinsic niche factors within hypoxic and perivascular regions play important role in maintaining and promoting stemness in GBM. For example, previous studies from Grassi *et al.* showed that Delta-like non-canonical Notch Ligand 1 (DLK1), expressed by tumor-associated astrocytes in these regions, acts as key regulator of stem-like properties⁹⁶. CD44 is a central mediator in these niches, as CD44-osteopontin signaling promotes stemness, and Johansson *et al.* further demonstrated that CD44 in the same regions is cleaved and interacts with Hypoxia-inducible factor (HIF)-2 α , thereby regulating hypoxia-induced stemness in both true hypoxic, but also in pseudo-hypoxic environments^{97,98}.

The continuing work to unravel stemness properties is clinically important, as GSCs or cells with stemness properties display intrinsic resistance to chemotherapy and radiotherapy. Mechanisms underlying this resistance include reduced proliferation rates, increased expression of drug-efflux transporters and increased reactive oxygen species (ROS) detoxification⁹⁹⁻¹⁰¹.

In Vitro Methods for Stemness Properties

In vitro approaches to investigate GSC properties include several experimental models. Cells are usually grown under controlled serum-free conditions, to avoid

unwanted differentiation and preserve stemness properties of cells. Media is supplemented with controlled growth factors, such as epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) to support their self-renewal and proliferative ability¹⁰². Measurements of stemness are performed using sphere-formation assays to assess self-renewal, radiation and chemotherapy resistance by colony formation assays, drug-efflux capacity through side-population assays and expression profiling of stem-cell markers^{103,104}. Together, these methods help functionally validate the stem-like behavior suggested by transcriptomic data.

Glial Cells

Brain cells can broadly be divided into neurons, glial, immune cells and vascular cells. Glial cells consist of oligodendrocytes, astrocytes and microglia and makes up to approximately 60% of the brain mass. The word “glia” means “glue” in ancient Greek, corresponding to their original definition of being the ‘glue of the brain’. The most abundant glial cell type of the brain is the star-shaped glial cell called astrocytes, consisting of approximately 20-40% of the total glial cell population in the brain¹⁰⁵. They have large, branched morphology, with a diameter reaching 150 μm consisting of intermediate filaments, in particular, glial fibrillary acidic protein (GFAP) forming the so-called astrocytic foot processes, allowing astrocytes to communicate with their surroundings¹⁰⁶. Astrocytic endfeet together with pericytes, are key structural components of the BBB, by enveloping endothelial cells and maintain and regulate BBB permeability by secreting factors such as bFGF and glial cell derived neurotrophic factor (GDNF) that tightens the BBB¹⁰⁷⁻¹⁰⁹. Additionally, astrocytes preserve brain homeostasis and optimal neuronal activity by monitoring the ion concentrations in interstitial fluids and keeping them constant by uptake or release of ions, as well as acting as metabolic support, releasing lactate during energy depletion. Astrocytes have historically been considered to primarily be a passive support cell for brain structure and neural functions, but increasing evidence shows that astrocytes play crucial roles in several neurological pathways, such as regulation of neuronal networks, detoxification and recycling of neurotransmitters.

Glial Development

Radial glia cells (RGCs), a type of neural stem cell (NSCs), in the ventricular zone (VZ), serve as a crucial common progenitor cell for neurons, astrocytes and oligodendrocytes. During early embryonic development, neurogenesis creates neuronal networks by producing neurons from NSCs, occurring in the VZ and subventricular zones (SVZ)¹¹⁰. Although RGCs initially generate neurons during the early stages of embryonic development, the gliogenic switch, after E11.5 in mice, start to produce intrinsic transcription factors SOX9, NFIA and NFIB that drives the glia towards an astrocytic lineage^{111,112}. Extrinsic factors, such as cytokines like CNTF and leukemia inhibitory factor (LIF) activate JAK/STAT pathways, further leading to activation of astrocytic genes such as GFAP and S100B and repression

of neuronal and oligodendrocyte genes (e.g. *Ngn1/2/3* and *olig1/2*) (Figure 1). Overall, there is a balanced and highly coordinated switch from neurogenesis, the formation of neurons, to gliogenesis, which creates the supporting cells such as oligodendrocytes and astrocytes in the developing brains. SVZ astrocyte like-progenitors arise embryonically from RGCs and persist through adulthood as a primary source of new glial cells¹¹³⁻¹¹⁵. These are located in the SVZ, and may under circumstances, such as injury, give rise to new astrocytes that can migrate to the region needing repair, and among other things, contribute to glial scar formation¹¹⁶⁻¹¹⁹.

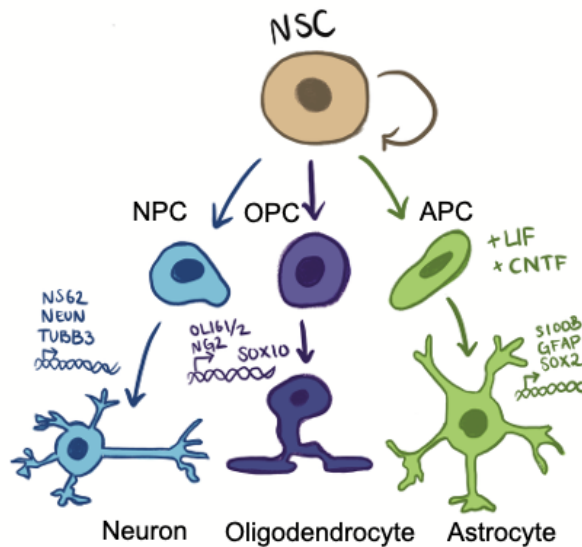


Figure 1. Illustration of development of neurons, oligodendrocytes and astrocytes.

Astrocyte Subtypes

The location, morphology and functions have given rise to the different astrocytic subtypes. Morphologically, astrocytes are classified as protoplasmic, fibrous or interlaminar. These can be present in different brain locations, such as cortex and cerebellum. Protoplasmic astrocytes are the majority of astrocytes found throughout the cortex. These have bushy, highly branched morphology, and provide metabolic support for neurons, ion homeostasis, and regulation of synaptic transmissions¹²⁰. In contrast, fibrous astrocytes are found along the white matter tracts and have longer straight processes and are in close proximity to the vasculature, their role is therefore thought to be highly involved in BBB maintenance¹²¹. Additionally, human and primates contain two subclasses called interlaminar and varicose astrocytes. Interlaminar astrocytes are located at the outermost part of the cerebral

cortex, and have long fibers extending into other layers of the cortex in, and express high amounts of GFAP, whereas varicose astrocytes also possess long foot processes similar to interlaminar astrocytes, but located in the deeper cortical layers, suggesting they might contribute to the complex cognitive functions in primates¹²².

More recent work, integrating both scRNA-seq and spatial transcriptomics, highlight astrocyte clusters specific to their anatomical location in the mouse brain^{123,124}, for example telencephalon (Astrocyte TE), non-telencephalon (Astrocyte NT), cerebellum, Bergmann glia or olfactory bulb astrocytes¹²⁵. Although several types of astrocytes have been found in different regional locations in human and murine brains, much of their diversity in transcriptional profiles remains to be explored¹²⁶.

Astrocyte Reactivity

Of particular importance to this thesis is the process of astrocyte reactivity. During injury or disease astrocytes undergo molecular, morphological and functional alterations called ‘reactive gliosis’. Astrocytes respond with increased proliferation and migration to the site of injury, surrounding the damaged area by growing thicker and longer processes¹²⁷. The generated surrounding scar tissue acts as a physical barrier to protect the remaining healthy brain. At present, it remains uncertain if astrocyte reactivity is restricted to certain subsets of astrocytes, or if it is a pan-astrocytic feature in response to disease. In general, reactive astrocytes display hypertrophy with increased expression of GFAP. Other markers commonly associated with reactivity are the upregulation of cytoskeletal markers Vimentin and Nestin, transcription factors such as STAT3, adhesion molecule CD44 and a broad range of inflammatory cytokines and chemokines. Broadly, astrocyte reactivity has been divided into neurotoxic or neuroprotective properties¹²⁸. However, astrocyte reactivity has proven not to be a unified molecular signature, but rather represents a diverse group of cells with different degrees of reactivity depending on the severity of the injury or microenvironmental cues^{129,130}. Recent advances in the field suggest classification based on their location and properties such as (i) proliferative, border-forming reactive astrocytes, that arise from periventricular neural progenitors in response to damage, or (ii) non-proliferative reactive astrocytes that retains tissue architecture, but respond and become locally reactive in response to disease or injury¹³¹. Based on these categorizations, proliferative astrocytes are suggested to gain non-canonical functions with increased interaction with immune and non-neural stromal cells, whereas non-proliferative local astrocytes retain their functionality and interaction with for example, synapses, neurons and blood vessels.

Astrocyte reactivity can be triggered either via a direct or indirect manner. After injury, astrocytes become activated through a diverse range of signaling, by sensing

damage associated molecular patterns (DAMPs) by pattern recognition receptors, mechanical stress, hypoxia or indirectly via TNF- α , IL-1 α and C1q crosstalk with microglia/macrophages¹³². Activation of astrocytes leads to increased signaling pathways of NF- κ B, STAT3 and transforming growth factor (TGF)- β , leading to astrocyte hypertrophy, proliferation and scar formation¹³³. The formation of the glial scar result in a protective barrier of healthy brain cells at the site of injury.

Astrocytes in CNS pathologies

Astrocytes have been extensively studied in central nervous system (CNS) pathologies, including, ischemic stroke, multiple sclerosis, traumatic brain injury, Alzheimer's, Parkinson's disease as well as amyotrophic lateral sclerosis and epilepsy. What is clear is that astrogliosis can be considered a spectrum, ranging from mild, reversible changes to more extended glial scar formation rather than binary categories. The reactivity results in loss- or gain-of-functions, and alterations also occur in their metabolic support (e.g. use of cholesterol and lactate), synapse formation, modulated immune cell interaction, and BBB maintenance. Gained functions include supporting axon regeneration, scar border formation and neurotoxin release. Recent advances of scRNA-seq and spatial transcriptomics display reactive astrocyte heterogeneity with overlapping gene programs that vary depending on disease context, disease progression, time after insult and CNS regions. For instance, inflammatory signaling (IL-1 β , TNF- α , complement C3) mediated by reactive astrocytes during acute infection or injury promotes immune defense and clears debris, whereas sustained neuroinflammation, leads to chronic neurodegeneration and synapse loss. However, most importantly, collectively we have learned that reactive astrocytes in the abnormal CNS secrete a diverse range of molecules ranging from chemokines, among them including C-C motif chemokine ligand (CCL)2, CCL3, CCL20, CXCL1, and cytokines such as IL-1 β , IL-6, TGF- β , TNF- α and interferon (INF)- γ , as well as complement proteins C3, C5, and complement factor B (CFB), and growth factors, e.g. VEGFA, FGF, BDNF and PDGF¹³⁴ (Figure 2).

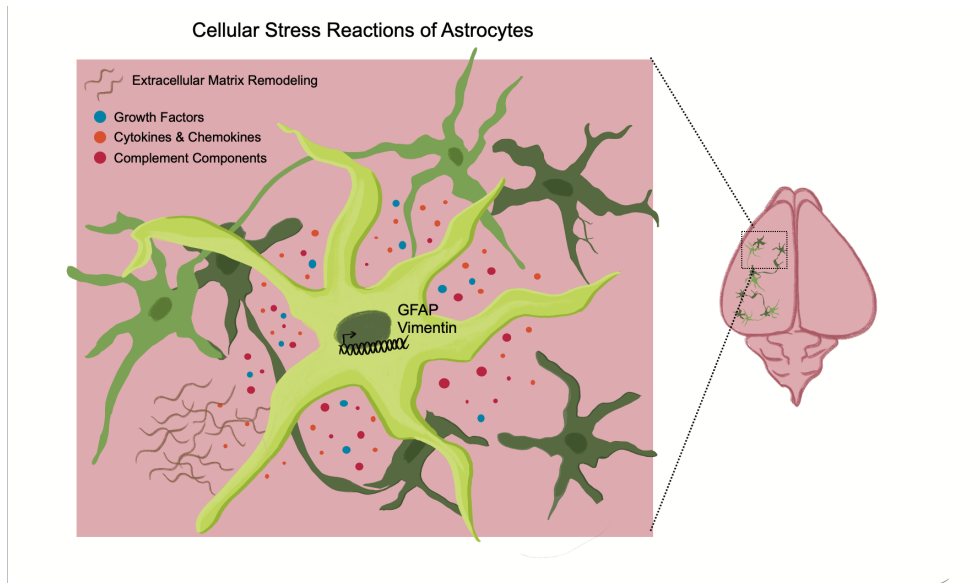


Figure 2. Illustration of local stress responses of astrocytes, which may be induced by CNS disease, damage, radiotherapy or tumors.

Astrocytes in the Context of Brain Tumors

Reactive astrocytes are known to limit inflammatory spread after stroke, traumatic injury, and other forms of CNS damages, a process critically dependent on STAT3-mediated signaling. In contrast, studies in mouse models of brain tumors demonstrate that neoplastic cells can hijack the same STAT3 pathway to suppress immune responses, thereby co-opting astrocytes to assisting in immune evasion of the tumors.

Astrocytes roles in hypoxia and radiation will be discussed in later chapters, as well as their relationship to tumorigenesis. This is an ongoing evolving field but it is clear that tumor cells and astrocytes are signaling bidirectionally, contributing to active disease. Astrocytes have been shown to play roles in both primary tumors and brain metastasis¹³⁵. Reactive astrocytes secrete high levels of factors such as $\text{TNF-}\alpha$, $\text{TGF-}\beta$ and IL-6 in response to CNS injury, and these molecules have shown to markedly enhance the *in vitro* proliferation of cancer cells, including brain-metastatic breast and lung cancer cells as well as primary brain tumors like GBM, elegantly summarized by Placone *et al.*¹³⁶⁻¹³⁸. Several studies conducting co-culture experiments have shown survival benefits or increased aggressiveness of cancer cells grown in co-culture, conditioned media or astrocyte-derived matrix¹³⁹⁻¹⁴⁴. Likewise, astrocytes contribute to chemoprotection in both primary and metastatic brain tumors. This effect has been detected in GBM cell lines, brain metastatic

breast, lung and skin cancers¹⁴⁵⁻¹⁴⁷. The current hypothesis is that cancer cells escape from chemotherapy-mediated cytotoxicity through regulation of calcium, through cell-cell contact gap junctions between astrocytes and tumor cells, for instance through connexin 43 (*GJA1*)¹⁴⁸. Astrocytes having pro-tumoral properties was ultimately shown by Perelroizen *et al.*, demonstrating that depletion of tumor associated astrocytes prolonged survival of mice, which was at least partially mediated by a lower influx of immunosuppressive macrophages¹⁴⁹.

How astrocyte reactivity, and their relative abundance, changes during tumor recurrence remains largely unexplored. Recurrent gliomas have been reported to display a higher reactive astrocyte scores^{65,150}. Consistently, a murine glioma-resection model showed that surgical injury induces a reactive astrocyte phenotype that fuels the regrowth of tumor¹⁵¹.

Overall, astrocytes appear to support tumor progression through different principal pathways. First, they promote direct survival of tumor cells by upregulating factors that enhance survival, especially under stressful conditions such as hypoxia, radiotherapy and injury. Second, they remodel the ECM to support tumor growth, and thirdly, they shape the immune microenvironment through extensive crosstalk with immune cells, particularly microglia and macrophages. Reactive astrocytes exist in dynamic, overlapping states, where the same functions might have protective or pathogenic roles depending on their context¹⁵². Targeting global astrogliosis is therefore challenging, but identification of specific signaling pathways, or temporal windows is considered more promising.

Transcriptomic Identification of Astrocytes

In experimental procedures, identification of astrocytes often relies on marker expression, such as GFAP. GFAP is expressed by virtually all reactive astrocytes, but may be absent in some resting astrocytes, and therefore has limitations as a pan-astrocytic marker. A combination of markers that are robustly expressed in astrocytes is therefore suggested for accurate detection of broader astrocyte populations. For instance, markers associated with the neural-glial switch, such as *FGFR3*, *S100B*, *FABP7*, *SOX9* and *BLBP* may be used, although not completely exclusive for astrocytes¹¹¹. Additionally, maturation markers such as *GLAST-1*, *AQP-4*, *GLT-1* and *ALDH1L1* are also suggested candidates¹⁵³. Some identification markers and stress-reactions are summarized in table 1.

Table 1. Identification and summary of astrocytic traits.

Cellular stage	Genes
Progenitor cell	<i>SOX9, FGFR3, S100B, BLBP, NFIA, S100A6, Nes, Fabp7</i>
Mature markers	<i>GLAST-1, AQP-4, GLT-1, ALDH1L1, GFAP, GJA1</i>
Reactivity markers	<i>GFAP, Vim, Nes, CD44, C3, C4b</i>
Biological trait	Reactivity-induced secretion
Complement	C3, C5
Angiogenesis	VEGFA, FGF, PDGF
Tumor cell growth	FGF, BDNF, PDGF
Pro-inflammatory signaling	IL-1 β , TNF- α , INF- γ
Immune suppression	IL-6, TGF- β , INF- γ
Immune requitement	CCL2, CCL3, CCL20, CXCL1, TNF- α
Tissue repair	CCL20, CXCL1, TGF- β
Hypoxic adaptation	CCL20, TNF- α

The Tumor Microenvironment

The TME is a complex ecosystem surrounding a tumor, including tumor, immune and stromal cells, blood vessels and the extracellular matrix. As the tumor starts to develop, it actively remodels its surrounding tissue at a molecular, cellular and physiological level to support its growth and progression¹⁵⁴. As highlighted by Truffi *et al.* the tumor microenvironment is “not just a silent bystander, but rather an active promoter of cancer progression”¹⁵⁵.

The Blood Brain Barrier

The BBB is a physical and chemical, highly selective, semi-permeable membrane. It is composed of endothelial cells, pericytes and astrocyte end-feet, that together separate the circulating blood from the brain tissue across blood vessels in most areas of the brain. Its main function is to act as a protective layer, preventing harmful substances or microbes to enter the brain, and at the same time maintain a stable environment for neurons to function properly. It is composed of tight junctions between endothelial cells, allowing only water and specific ions to pass whereas it prohibits the majority, around 98% of small molecules, and larger molecules like growth factors and antibodies to pass^{156,157}. The BBB therefore remains one of the largest obstacles in delivering drugs to GBM, and it is hypothesized that glioma cells residing at the tumor border are protected from anti-cancer agents due to the intact vasculature, whereas the center of tumors often exhibit an impaired BBB with more allowance for drug permeability^{156,158}.

Hypoxia

In tissues, oxygen plays a crucial role of maintaining cellular viability. The absorption of O₂ into the body tissues occurs in the gas-liquid interface of our alveoli and are thereby the major limiting factor of oxygen distribution. Physiological level of oxygen varies depending on the tissue but has been reported between 14- to 2.5%, from highly oxygenated arterial blood in contrast to lower oxygenated organs such as the brain or skeletal muscles. Overall, factors regulating the oxygen levels in

various tissues are based on their oxygen consumption rate, vascularization and blood flow¹⁵⁹. Hypoxia occurs when there is insufficient oxygen supply to the tissues, resulting in impaired cellular homeostasis, often associated with pathological conditions such as inflammation, bacterial infection, wounds, cardiovascular diseases and cancer. Therefore, our bodies have developed systems to regulate and maintain adequate oxygen levels, through responses such as increased circulating red blood cells, blood vessel growth, ventilation and cardiac output to compensate for lack of oxygen¹⁶⁰. Cellular hypoxia can be temporary, due to imbalance between metabolic requirements and oxygen supply, or permanent because of vascular damage, inflammation or tissue swelling. In addition, different degrees of oxygen deprivation elicit distinct cellular responses. Mild hypoxia activates survival programs focusing on enhanced angiogenesis and altered metabolism, whereas severe hypoxia leads to cell cycle arrest, TP53 activation, autophagy and induction of apoptotic and necrotic pathways. Hypoxia can either be transient, if tissues regain physiological oxygen concentration (physoxia), or chronic if it permanently alters the microenvironment or vessels.

Both normal and tumor cells have adapted mechanisms that respond to variations in oxygen levels, through a transcriptional program mainly regulated by HIFs.

Hypoxia-Inducible Factors

HIFs are heterodimeric transcription factors, consisting of two basic helix-loop-helix proteins (HIF- α and HIF- β) that are continuously expressed in all cell types. Under adequate physiological oxygen concentrations (normoxia), the oxygen-sensitive HIF- α subunit (consisting of three, HIF-1 α , HIF-2 α and HIF-3 α isoforms) has a short half-life. HIF- α becomes hydroxylated by oxygen-dependent prolyl hydroxylase domains (PHDs), resulting in HIF- α to be recognized by Von Hippel Lindau tumor suppressor protein (pVHL), a component of the E3 ubiquitin ligase complex. In this way, in the presence of oxygen pVHL targets HIF- α for degradation via the ubiquitin-proteasome pathway in presence of oxygen. However, in the absence of oxygen PHDs are no longer capable of hydroxylating HIF- α for proteasomal degradation, leading to accumulation and translocation to the nucleus and dimerization with HIF-1 β . The heterodimeric HIF-complex binds to hypoxia response elements (HRE) in gene promoters and increases expression of target genes.

HIF-signaling was collectively identified in the 1990s from Gregg Semenza, Peter Ratcliffe and William Kaelin's research groups. For their discoveries, of how cells sense and adapt to oxygen availability, they were jointly awarded the Nobel Prize in Medicine in 2019¹⁶¹.

Cellular Response to Hypoxia

Under physiological O₂ levels, our cells generate high amounts of energy in the form of adenosine triphosphate (ATP) from glucose, using cellular respiration. This includes two phases, firstly glycolysis, an oxygen-independent pathway which converts glucose to pyruvate, which is imported to mitochondria for oxidative phosphorylation, generating high amount of ATP for our cells. Cells in oxygen-deprived environments, and often tumor cells, uses only the oxygen-independent process called anaerobic glycolysis, which converts glucose to pyruvate, followed by conversion to lactate instead of performing oxidative phosphorylation. Tumor cells can rely on this metabolic reprogramming for energy even when oxygen is present, termed aerobic glycolysis, a phenomena firstly described by Otto Warburg in early 20th century and later recognized as the “The Warburg effect”¹⁶². The accelerated glucose metabolism is conducted by upregulation of glucose transporters such as GLUT1. The process is often initiated by stabilizing HIF-signaling under normoxic conditions, called pseudohypoxia. Mutations in certain tumor suppressor genes, such as for instance VHL, MDM2 or TP53 leads to abnormal regulation of the hypoxic signaling and leads to accumulation of HIF proteins even under physiological oxygen concentrations¹⁶³. The overall cellular response of normal, and pathological cell responses to hypoxia includes the transcription of genes containing the HRE and are involved in glucose metabolism, transcriptional regulation, erythropoiesis, angiogenesis, pH regulation and cell survival. Measurable markers of hypoxia can therefore include for instance, *GLUT1*, *LDHA*, *HK2*, *BHLHE40*, *EPO*, *VEGFA* and *CA9*¹⁶⁴⁻¹⁶⁶.

Tumor Hypoxia

Hypoxia is a defining feature of GBM, as well as many other solid tumors. It arises due to rapid and infiltrative growth of tumor cells outgrowing the capacity of the aberrant tumor vasculature and oxygen supply, resulting in a larger distance with approximately 200 µm away between vessels to cells, ultimately creating niches of chronic hypoxia and in severe cases necrosis. Endothelial cells become abnormal from interaction with the TME, as well as exposure to damaging agents such as anti-cancer drugs, resulting in leaky, non-functional vessels and spatially heterogenous oxygen levels within the tumor mass. Even if tumor hypoxia is highly inter- and intra-variable within tumors, a higher degree of tumor hypoxia is associated with decreased survival of patients in various cancer types¹⁶⁷.

Tumor and stromal cells can adapt to the lower oxygen environment by activating transcriptional programs involved in metabolism, angiogenesis, survival and extracellular matrix (ECM) remodeling primarily driven by HIFs. These adaptations enable tumor cells to withstand oxygen scarcity while reshaping the surrounding microenvironment. Hypoxic niches, associated with perinecrotic and

pseudopallisading necrosis is one of the major characteristic distinguishing GBM from lower grade gliomas. Furthermore, PTEN and Tp53 mutations frequently found in GBM are both associated with adaptation to hypoxia through increased glycolysis via PI3K/Akt signaling and glucose transporter regulation¹⁶⁸. Additionally, PDGF-signaling has been shown to stabilize HIF-1 α ¹⁶⁹. Hypoxic zones enrich for populations of glioma cells exhibiting stem-like features (dedifferentiation and upregulation of stemness markers OCT4, Nestin, CD133 and SOX2¹⁷⁰) and are highly linked to radioresistance^{171,172}. Therefore, GSC have been associated with certain niches of the TME, suggesting that factors in those environments may promote the stemness properties of glioma cells^{96,98}. Already in 1953 Gray *et al.* published the relationship of oxygen availability and effectiveness of radiotherapy, showing that tumors cells responded approximately three times higher to radiotherapy in normoxia compared to hypoxia¹⁷³. The spatial distribution of oxygen partial pressure (pO₂) is therefore a critical factor when it comes to radiotherapy, as radiosensitivity is strongly influenced by tissue oxygen tension and tumor cells residing in adequately hypoxic or severely hypoxic environments therefore exhibit higher resistance to radiotherapy¹⁷⁴. This is partially because tumor cells in hypoxic niches have an imbalanced electron flow in the electron transport chain due to low oxygen, generating a higher amount of ROS in those areas^{175,176}. Tumor cells avoid harmful effects from ROS by upregulating ROS scavenging enzymes, allowing them to survive and proliferate under hypoxic conditions¹⁷⁷. Under normoxic conditions, radiotherapy works by generating high amount of ROS when molecular oxygen reacts with radiolysis products of water. These reactive compounds can interact with DNA to form stable peroxyl adducts that are harder for cells to repair¹⁷⁸. Radiation under low oxygen concentration produces fewer ROS and therefore causes less radiation-induced DNA damage. Cells in these hypoxic niches are further protected by upregulated antioxidants such as glutathione and thioredoxin, which quench ROS, and any DNA damage that does occur can be repaired more efficiently than compared to normoxia¹⁷⁹⁻¹⁸².

Stromal cell response to hypoxia

Beyond tumor cells, hypoxia influences multiple non-neoplastic compartments of the GBM microenvironment, including astrocytes, endothelial cells, and immune cells, including microglia, infiltrating macrophages and T-cells. Astrocytes, in hypoxic stress, which has mainly been studied in ischemic brain injuries or stroke, alter their behavior and demonstrate a reactive phenotype associated with hypertrophy, and increased expression of GFAP, S100B, vimentin and neurotrophic factors¹⁸³. In the context of GBM, it has been shown that hypoxic astrocytes release factors, such as CCL20 which amplify the HIF-1 α -signaling in glioma cells, thereby boosting malignancy by helping glioma cells adapt better to low oxygen

environments¹⁸⁴. Chronic hypoxia therefore drives astrocytes to secrete pro-tumorigenic ligands, and support glioma stem cell growth¹⁸⁵.

Moreover, immune cells, such as infiltrating macrophages, are recruited to hypoxic niches by chemokine attractants (e.g. CCL8 and IL-1 β), and are strongly shaped by hypoxic niches, and express immunosuppressive cytokines (IL-10, IL-4), immunosuppressive markers (CD206 and Arginase-1) and mediators of immune tolerance (PD-1/PD-L1)¹⁸⁶. The immunosuppressive landscape is further promoted as hypoxia pushes T-cells towards a dysfunctional and exhausted state. Increased VEGF expression in hypoxic areas reduce expression of adhesion markers (ICAM-1 and VCAM1) resulting in decreased overall infiltration of T-cells. The T-cells that do enter into the TME, are immediately exposed to a cocktail of immunosuppressive cytokines, such as IL-10 and TGF- β , and immune-regulatory ligands (PD-L1 and CTLA-4) expressed on glioma cells and M2-skewed macrophages, resulting in decreased T-cell response and exhaustion^{187,188}.

Overall, tumor hypoxia is a defining hallmark of the GBM TME and a key driver of stromal and immune cell reprogramming. Reduced oxygen tension stabilizes HIF-1 α and HIF-2 α , altering the behavior of tumor cells, resident brain cells, and infiltrating immune cells. Low oxygen drives astrocytes and other stromal cells into reactive, tumor-supportive states by secreting cytokines, chemokines, and extracellular matrix proteins that enhance tumor cell survival, invasion and therapy resistance. At the same time, hypoxic niches create a supportive environment that helps maintain glioma stem cells. Hypoxic stress increases tumor genetic instability by downregulating DNA-repair mechanisms, thereby elevating their mutational burden and promoting aggressive radioresistant clones¹⁸⁹. Several studies have shown that GBMs with mesenchymal signatures display strong hypoxic gene expression or high levels of immune infiltration, suggesting that hypoxia or recruited inflammatory cells, such as M2-like macrophages, may function as niche factors that supports mesenchymal GBM states^{72,190,191}. Inflammatory cytokines such as TNF- α have also been shown to sustain hypoxic signaling through stabilization of HIF-1 α ¹⁹². Building on the GBM cell-state framework, Neftel *et al.* refined the mesenchymal classification by identifying two distinct mesenchymal programs, a hypoxic dependent signature (MES2) and a hypoxia-independent signature (MES1)⁶³. Zhang *et al.* further demonstrated a spatial association between tumor-associated macrophages and hypoxic transcriptional programs, illustrating how inflammatory cues may cooperate with hypoxia to drive the MES2-like state¹⁹³.

Radiation Induced Alterations of the Tumor Microenvironment

Glioblastoma is treated primarily through surgery, radiotherapy and chemotherapy. Radiotherapy is typically performed with an accumulative dose of 60 Gray (Gy), divided over 30 days x 2 Gy. The introduction of radiotherapy in GBM treatment has significantly prolonged the life of patients, however, treatment resistant tumors almost always relapse. Recurrent tumors appear in the same location as the primary tumors that received high radiotherapy treatment¹⁹⁴, suggesting that some tumor cells are capable of escaping the initial treatment, and give rise to treatment-resistant relapsed tumors. The effect of radiotherapy on tumor cells has been extensively studied, however, radiotherapy does not merely effect tumor cells, but all stromal cells in the TME receive the same dose therapy as the malignant cells. Radiotherapy has been shown to generally remodel the brain microenvironment in a pro-tumoral setting, specifically shown by Fletcher-Sananikone *et al.*¹⁹⁵, Duan *et al.*¹⁹⁶, Ji *et al.*¹⁹⁷ and Berg *et al.*¹⁴⁰ that pre-irradiated mouse brains develop gliomas faster with decreased survival. This gives direct evidence that radiotherapy even of a healthy brain creates a pro-tumor environment that better supports GBM growth. Continued understanding of radiotherapy effects on the stromal and ECM compartments opens for targeted therapy that reverts the pro-tumoral effects mediated by stromal cells.

Tumor Cell Responses to Radiotherapy

Radiotherapy induces cell death through various mechanisms. It causes a direct physical damage to exposed cells and areas through single- or double-stranded DNA breaks, as well as ECM remodulation via fragmentation or crosslinking of proteins. Furthermore, radiation leads to increased amount of ROS formation, both directly through ionizing water molecules, and through damaging mitochondria. Higher amounts of ROS result in injuries to cellular membranes (lipid peroxidation), protein malfunctions and further damages to DNA^{198,199}. Disruption of the redox homeostasis, and increased oxidative stress from free radicals, often results in cellular senescence or death. However, ROS production has dual roles in tumorigenesis, as it can induce tumor cell death, but also increase genomic instability and epigenetic adaptations that may fuel tumor growth^{200,201}. Due to advances in imaging and planned delivery dosage, radiotherapy can more selectively target tumor areas, with limited effects on surrounding healthy tissue²⁰². In general, healthy cells exposed to radiotherapy recover more easily, due to functioning repair mechanisms that are downregulated in malignant cells. Even so, it has been reported that patients with high dose RT can experience long-term cognitive impairments^{203,204}, due to the radiotherapy exposure that healthy tissue receive during treatment.

Radio-resistance of recurrent cancer cells is a common and significant consequence after radiotherapy, resulting in subsequent treatment failures in relapsed tumors²⁰⁵. Cancer cells can overcome radiation-induced damage created with ROS through dysregulated antioxidant systems and increased expression of ROS-scavenging enzymes as well as removal of damaged intracellular organelles by increased autophagy²⁰⁶. Targeting these systems to modulate the redox homeostasis and making malignant cells more radiosensitive are under current investigations and shows promising results in preclinical studies²⁰⁷.

Stromal Cell Responses to Radiotherapy

Apart from tumor cells, adjacent non-tumor cells including immune cells, such as microglia/macrophages, vasculature (endothelial cells and pericytes), glia cells (astrocytes, oligodendrocytes) and neurons receive the same amount of radiation exposure as malignant cells during treatment. Recent research has shifted the focus to stromal cell responses to radiotherapy, and how irradiation (IR)-induced responses may help fuel the tumor growth and recurrence²⁰⁸. Understanding how stromal cells contribute to aggressive tumor behavior provides new routes to targeting therapy-related resistance rather than targeting tumor cells themselves.

Vasculature

Radiation exerts opposing effects on normal and tumor-associated vasculature. It damages the vascular networks, inducing endothelial senescence, inflammation, immune cell infiltration and hypoxia-driven revascularization²⁰⁹⁻²¹¹. Although vascular disruption may initially seem beneficial by restricting nutrient supply, reduced oxygen availability diminishes radiation-induced ROS, thereby limiting the effects of radiotherapy itself²¹². Additionally, damaged vasculature results in increased hypoxic regions associated with glioma stemness and progressive disease, as well as challenges therapeutic drug delivery. This paradox has been illustrated in pre-clinical studies, where inhibition of angiogenesis alone can improve survival, but combining it with radiotherapy abolishes the radiation benefit²¹³. Another additional complexity is highlighted by pericytes role in GBM vasculature. A study from Zhou *et al.* showed that GBM has increased pericyte coverage, which was correlated with worse post-radiotherapy survival, and pericyte depletion increased drug effusion into tumors²¹⁴. Theoretically, targeting the blood tumor barrier, could therefore potentially act as a promising future treatment strategy to increase drug deliveries. However, our recent collaborative publication, from Braun *et al.* showed tumor-suppressive properties of pericytes, and genetic ablation was associated with tumor progression and shorter survival, mediated by an increased immune-suppressive environment via recruitment of tumor-associated macrophages²¹⁵. Collectively, the above findings illustrate the inherent difficulty in using tumor

vasculature as a therapeutic target and emphasizes the necessity for further mechanistic understanding to overcome these dual complexities.

Immune Cells: Microglia and Macrophages

The immune landscape of the brain is altered in the presence of brain tumors and is further reshaped by radiotherapy. Microglia, the resident immune cells of the brain, together with bone-marrow-derived macrophages, are highly present in brain tumors and are estimated to representing around 30-50% of cells. These myeloid cells exist along a polarization spectrum commonly described as ranging from pro-inflammatory (M1) to immunosuppressive (M2) phenotypes. In glioblastoma, tumors typically skew these cells toward a pro-tumorigenic, immunosuppressive state^{216,217}, although it is difficult to divide tumor associated macrophages along the M1-M2 axis²¹⁸.

Recent studies show that tumor-associated macrophages accumulate at the tumor margins²¹⁹, where they promote glioblastoma stemness, or a mesenchymal-like phenotype of GBM cells²²⁰, all associated with worse prognosis. Although microglia and macrophages become activated in response to tissue damage induced by radiotherapy, and tumor recurrence is associated with a shift from microglia-dominated to bone marrow-derived macrophage populations^{221,222}, it remains unclear how ionizing radiation modulates the balance between pro- and anti-tumorigenic functions of these cells. However, recent studies indicate that radiation favors M2-like polarization through the upregulation of glutamine synthetase, and conditioned media from irradiated microglia enhance glioma aggressiveness^{223,224}. Irradiated glioma cells themselves have shown to indirectly drive M2 phenotypes²²⁵, although it cannot be excluded that the higher presence of M2 after radiotherapy isn't due to disappearance of the M1 phenotype²²⁶. Preclinical studies, showed that inhibition of myeloid recruitment to tumors enhances radiotherapy responses^{221,227}. Interestingly, radiotherapy of microglia also leads to an altered interaction with other stromal cells in the tumor. Factors secreted by irradiated microglia support tumor cell growth by promoting gliosis from astrocytes²²⁸. Together, these findings suggests that radiotherapy directly, or radiation-induced bystander effects may contribute to a pro-tumorigenic environment mediated by microglia/macrophages.

Astrocytes

Studies from Monje *et al.* showed that following radiotherapy, gliogenesis is maintained to a much greater extent compared to neurogenesis. The latter being strongly influenced by extrinsic factors such as inflammatory mediators as NPCs could generate neurons after irradiation *in vitro*, but not *in vivo*^{229,230}. Importantly, blocking inflammation restored neurogenesis²³¹. These findings indicate that inflammatory responses to radiotherapy suppresses neurogenesis far more than

gliogenesis, and that glial cells have more adaptive properties to pathological or stressful conditions²³².

Another contributing factor for altering the neuron-glial balance may be that the CNS is highly vulnerable to oxidative stress, which is amplified by radiation-induced ROS^{233,234}. Oxidative stress persists in irradiated rodent brains, motivating antioxidant strategies such as ROS scavenging to promote radio-protectivity by, for example, antioxidant enzyme superoxidase dismutase (SOD)²³⁵. Interestingly, irradiation substantially reduces the neuron-to-astrocyte ratio in wild-type mice, whereas the ratio is preserved in SOD-deficient animals²³⁶. The enrichment of astrocytes in SOD knockout mice may support neuronal survival through redox regulation, although the functional significance of the astrocyte enrichment remains unclear. It is therefore intriguing to question whether radioprotective properties of astrocytes may also contribute to survival in other pathological settings, such as post-radiotherapy in GBM patients.

As previously mentioned, disruption of the BBB is a common response to radiation-induced brain injury. Within 24 hours of irradiation, astrocytes contribute to increased vascular permeability followed by a shift toward antioxidant and reactive states²³⁷. Astrocyte-endothelial communication plays a central role in radiation-induced BBB dysfunction, where endothelial senescence and dysfunction following irradiation has been linked to pro-inflammatory cytokines, such as TNF- α that can be released by reactive astrocytes^{238,239}. Indeed, using anti-TNF- α treatment abrogated radiation-induced astrogliosis and BBB-damage²⁴⁰. Additionally, Markarian *et al.* showed that radiation increased astrogliosis and neuroinflammatory cytokines such as TNF- α and IL-1 α . Here, an increased expression of complement proteins C1q and C3 was also observed, which were associated with radiotherapy-induced cognitive deficits often seen in patients with radiotherapy to the CNS²⁴¹. Particularly, C3 was increased in astrocytes already 2 hours post RT, and persisted up to 4 weeks after radiation exposure. Furthermore, some studies indicate that astrocyte activation after irradiation depends on microglial signals, including prostaglandin E2 (PGE2) and C1q^{228,241}. This suggests that there is an early inflammatory response of glial cells, that promotes a partially complement-mediated microglia-astrocyte proinflammatory crosstalk post radiotherapy that short-term may help removal of damaged tissue, but that persists in the brain several weeks after treatment with unknown long-term effects.

Overall, normal human astrocytes show robust repair responses to DNA damage induced by radiotherapy and are resilient up to single doses of 10 Gy of X-rays²⁴². Astrocytes exposed to cellular stress, such as radiotherapy, undergo a process called reactive astrogliosis²⁴³. This can be initiated either by single, or fractionated radiotherapy^{228,244}. Although research on radiation-induced alterations of astrocytes in GBM is still limited, several studies have shown the following; (i) *In vitro* co-

culture experiments of astrocytes with glioma cells increase the radio-resistance of GSCs^{140,245}, for instance, by releasing soluble factors, such like DLK1 to promote stemness characteristics of GBM cells⁹⁶ (ii) pre-irradiated astrocytes can remodel the extracellular matrix, as demonstrated by Berg, *et al.*, who showed that IR-induced expression of Transglutaminase 2 (TGM2) enhances glioma stemness and radio-resistance¹⁴⁰, and (iii) IR-induced astrocytic senescence leads to the secretion of cytokines such as TNF- α and IL-6 which increase glioma CXCL1 expression and promotes tumor progression by recruitment of myeloid cells¹⁹⁷.

Glial Scar and SASP

Cancer-associated fibroblasts (CAFs) are key components of the TME, contributing to cancer progression and supporting immune evasion. CAFs display intrinsic resistance to conventional radiotherapy doses, yet ionizing radiation can alter their secretory programs, and thereby modulate tumor-stroma and tumor-immune interactions²⁴⁶. A recent study, from Watson *et al.* showed that post-radiotherapy relapsed tumors exhibit dense fibrotic scarring. Fibrotic regions, caused by TGF- β signaling, were identified as pro-survival niches that sheltered residual glioma cells, promoted dormancy and impaired immune surveillance²⁴⁷.

Glial scar formation is a usual response from fibroblasts and astrocytes consequent to tumors or radiotherapy. Both fibroblasts and astrocytes contribute actively to the formation of glial scar in response to CNS injury, isolating the damaged area from healthy tissue. Both cell types have shown to engage in SASP after radiotherapy. The precise mechanism of how SASP contributes to tumorigenesis remains unknown, but several studies have confirmed changes in various soluble factors, growth factors, chemokines and cytokines, such as TGF- β , TNF- α , CCL2, IL-6 and IL-8, as well as ECM remodeling all participate in the response^{248,249}. This creates a chronic inflammatory profile that may additionally fuel the tumor recurrence. A promising way to target this SASP-mediated tumor promotion is the usage of senolytic drugs, specifically targeting senescent cells remaining after radiotherapy. *In vivo* models targeting senescent cells with an active *CDKN2A* promoter after radiotherapy, showed increased survival of glioma-bearing mice when senescent cells were ablated prior to implantation of glioma cells¹⁹⁷. Further investigation of which cells in the brain were actively expressing senescence markers in response to radiotherapy revealed that astrocytes were the main cell type, together with OPCs experiencing senescence in response to radiotherapy.

Overall, IR-responses of stromal cells includes an increased inflammatory landscape of tumors, with altered angiogenesis, fibrosis, glial scar formation and ECM remodeling, as well as shifts in cellular composition between immune and stromal cells (Figure 3). Although the majority of the tumor is eradicated in response

to radiotherapy, IR-induced senescent stromal cells may still secrete factors that contributes to fuel tumor regrowth from surviving tumor cells.

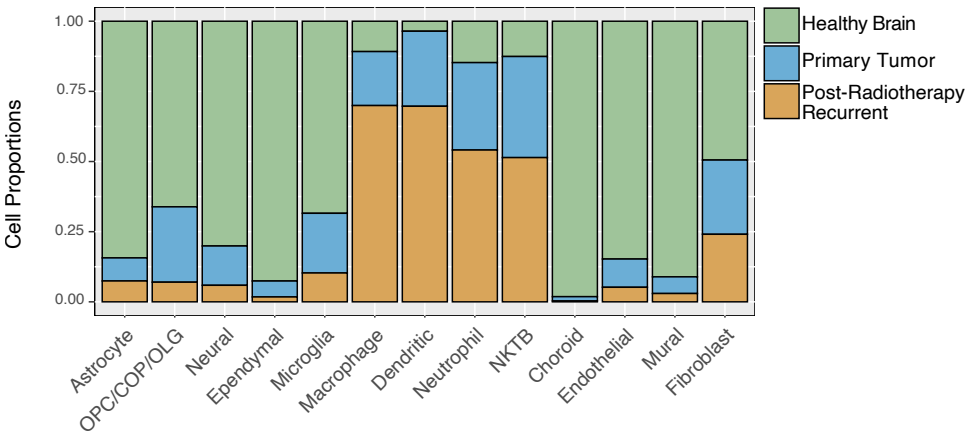


Figure 3. Supplementary figure 2, from Paper I, displaying the frequency of non-malignant cells between healthy brain tissue, primary and post-radiotherapy tumors.

The Complement System

The complement system is a crucial component of the innate immune response, comprising of more than 30 circulating and membrane-bound proteins that works together to maintain tissue homeostasis²⁵⁰. Beyond serving as one of the body's first line defense against pathogens, the complement system plays an important role in clearing apoptotic cells and cellular debris, thereby preventing chronic inflammation and supporting tissue repair. Once activated, complement proteins enhance inflammatory signaling, opsonizing targets to facilitate recognition and phagocytosis by immune cell, and can directly kill susceptible cells through formation of the membrane attack complex. The system is activated through three major pathways, the classical, lectin or alternative pathway, each triggered by distinct molecules, but all converging on a central amplification cascade to generate the same key effector molecules²⁵¹.

Activation of the Complement System

The classical pathway gets activated when Fc-regions of antibodies, or apoptotic cells binds to C1q, the first component of the C1-complex (C1q, C1r and C1s). It can also be activated independently of immune complexes, by C1q binding to pentraxins, both scenarios resulting in conformational changes activating C1q-associated proteases C1r and C1s. The lectin pathway on the other hand, gets triggered when mannose-binding lectin or ficolins bind to "foreign" carbohydrate patterns such as mannose sugars on pathogen surfaces, triggering activation of associated proteases in a manner similar to C1 activation. Activation of these associated proteases lead to a proteolytic cascade forming a C3-convertase. The alternative pathway in comparison is continuously active at low levels through spontaneous hydrolysis of C3, leading to a conformational change and association with complement factor B, allowing cleavage by factor D to also form the alternative pathway C3 convertase. Therefore, all three pathways lead to formation of C3-convertases, leading to cleavage of C3, generating C3a and C3b (graphical illustration in Figure 4). C3b binds to cellular membranes, acting as an opsonization molecule, or allows continued complement activation by binding further components of the complement system, creating C5-convertase, with cleavage products the pro-inflammatory peptide C5a and C5b, which can form the membrane attack complex consisting of C5b6-9, where multiple copies of C9 forming a membrane-piercing pore²⁵². C3a and C5a on the other hand, acts as anaphylatoxins,

and are generally considered pro-inflammatory as they recruit more immune cells by binding to their receptors C3aR and C5aR1/C5aR2, respectively.

Healthy cells are generally protected against complement-mediated cytotoxicity (CDC) through expression of complement regulators, such as CD35, CD46, CD55, and CD59, as well as by serum protein inhibitors of complement activation, such as C1 inhibitor, factor I (FI), factor H (FH) and C4BP. Relative absence of these regulators in comparison to abundance of initiating factors, especially for the spontaneously activating alternative pathway, can lead to uncontrolled activation²⁵³.

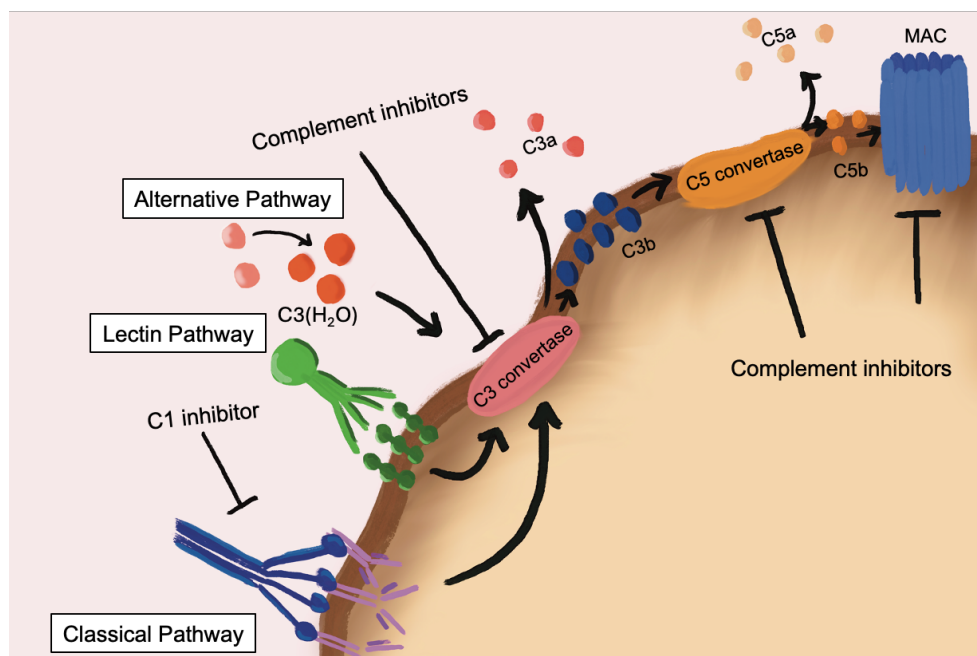


Figure 4. Graphical summary showing activation of the complement system.

Complement in Cancer

Dysregulated complement system activation is present in several pathological conditions, ranging from autoimmune and neurodegenerative diseases to cancer. Under physiological conditions, most circulating complement proteins are produced and released into circulation by the liver. However, recent discoveries show that various cell types can express complement proteins, resulting in local complement expression and activation. Furthermore, several components have shown to display non-canonical functions, independent of the complement cascade. The complement system is a double-edged sword in the TME²⁵⁴. It can contribute to anti-tumor immunity in certain contexts, by opsonization (C3b), targeting tumor cells for

phagocytosis, immune activation of T-cells mediated via C3a/C5a, and complement-dependent cytotoxicity via formation of membrane attack complex MAC²⁵⁵. Nevertheless, many tumors upregulate complement inhibitors and can escape complement-mediated killing. There is growing evidence that complement is chronically activated in many tumors. As previously mentioned, tumor-promoting inflammation is a hallmark of cancer and instead of eliminating cancer cells, this persistent low-grade inflammation can promote tumor progression via immune suppression, inflammation, angiogenesis and metastasis²⁵⁶.

Complement activity within the TME can originate from multiple cellular sources, including immune cells, stromal populations, and the tumor cells themselves. Tumor cells can produce complement components, and through autocrine C3aR- and C5aR-signaling activate PI3K/AKT pathways that promote proliferation and survival²⁵⁷. Clinically, high expression of C3 and C5 correlates with reduced survival across several cancer types and patient cohorts. *In vivo* studies further demonstrate that genetic deletion or pharmacological inhibition of C3a/C3aR or C5a/C5aR signaling improves survival in diverse murine cancer models, including lung, breast, colon, melanoma, pancreatic, leukemia and ovarian cancers^{255,258-260}. Most evidence highlights modulations of the immune microenvironment, leading to immunosuppressive and immune evasion. More specifically, C5a/C5aR-signaling has been linked to tumor growth by suppressing CD8⁺ T-cells, and increased myeloid-derived suppressor cell populations²⁶¹. In a clear-cell renal carcinoma model, a study from Roumenina *et al.* showed that tumors can hijack C1q from macrophages to promote their own growth via classical complement activation²⁶². Also here, mice deficient in central components, such as C1q, C3 or C4 had less tumor growth. Interestingly, the C3-depleted mice were almost completely protected from tumor progression.

However, findings are not fully consistent across all models. Some reports indicate that blocking of C3a and C5a signaling can lead to radiotherapy resistance, suggesting that these anaphylatoxins may also contribute to effective anti-tumor responses under certain conditions. Radiotherapy itself increases transcription and activation of complement proteins locally within tumors, and complement appears to participate in the early inflammatory and immunogenic response following radiation-induced tumor cell death²⁶³. However, radiotherapy in combination with C3a inhibition has also demonstrated enhanced natural killer (NK) infiltration and increased survival in a pancreatic mouse model²⁶⁴. Additionally, Beach *et al.* showed improved radiotherapy response in a colorectal cancer model when treated with a C5aR1 antagonist in an immune cell independent manner, specifically targeting tumor cell death in a cell-intrinsic way without affecting the normal intestine²⁶⁵. This shows that the role of complement in cancer can be context-dependent to the tumor type²⁵⁵.

Complement in brain tumors

Morgan *et al.* emphasized that astrocytes are immunologically active cells, engage in cytokine mediated signaling, respond to chemotaxis and are capable of small particle ingestion, similar to phagocytosis. It was firstly shown in 1987 by Lévi-Strauss and Mallat that astrocytes are capable of producing complement factors, C3 and factor B. Since then, it is shown that astrocytes could express and secrete all the proteins involved in the classical, alternative and terminal complement pathways. Under basal conditions, most components were secreted only at low levels, but exposure to INF- γ or other inflammatory cytokines, such as TNF- α and IL-1 β , markedly increased their expression²⁶⁶. Since then, it has been shown that several complement components, for instance, C1q, C3, C3a, and C5aR increase in the brain after exposure to radiotherapy²⁶⁷, highlighting their potential involvement in the injured or GBM treated brains.

In the context of brain tumors, regulation of complement activity is understudied, compared to its growing scrutiny in neurodegenerative diseases and dementia^{268,269}. Astrocytes are a major source of C3 production in the injured CNS, and C3 is widely recognized as a marker of reactive astrocytes in neurodegenerative diseases. Although it still remains unexplored if tumor-associated astrocytes also upregulate C3 expression in the context of glioblastoma, it has been demonstrated that C3 is highly upregulated in medulloblastoma, where C3a activates tumor-associated astrocytes to secrete pro-inflammatory cytokines such as TNF- α and IL-6, ultimately supporting tumor growth²⁷⁰. Earlier studies from Barnum and colleagues similarly reported C3 expression in astrocytomas and showed that it is regulated by cytokines including IL-1 β , TNF- α and INF- γ ^{271,272}.

Evidence for complement involvement in glioblastoma is currently emerging²⁷³. A study by Bouwens *et al.* found elevated serum levels of C1q in GBM patients, together with C3 deposition in both necrotic and non-necrotic regions of tumors²⁷⁴, suggesting that glioblastomas may locally produce or activate complement components. High C1q expression is correlated with worse patient survival and is associated with an M2-like macrophage phenotype within the GBM microenvironment²⁷⁵.

Collectively, these findings underscore the complexity and context-dependent nature of complement activity within the TME (Figure 5). Complement can either restrict or promote tumor progression depending on its cellular source, the specific signaling engaged, and the broader immunological context. In brain tumors, complement biology remains relatively unexplored, highlighting the need for further research to clarify its contributions to tumor growth, immune modulation and treatment response.

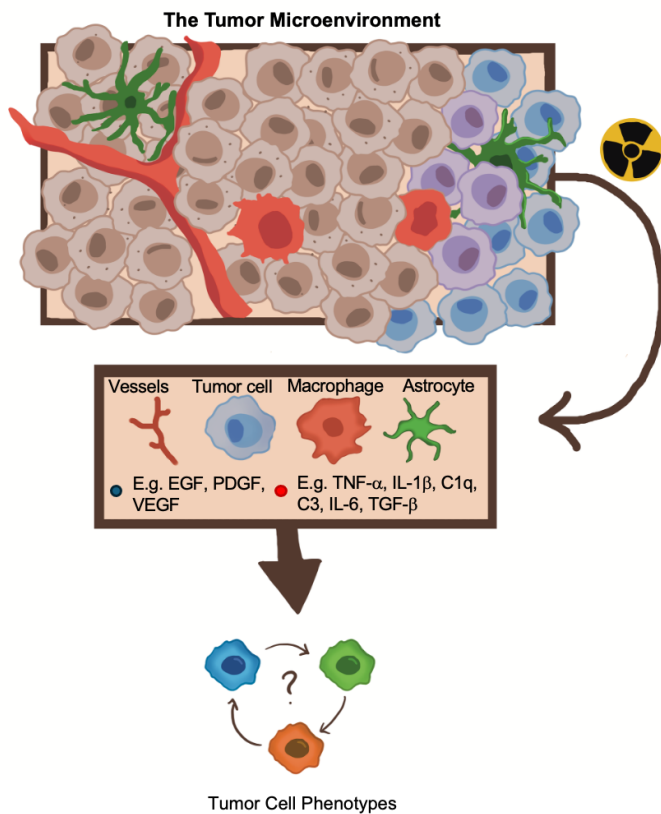


Figure 5. Summarizing the overall aim of this thesis to investigate how hypoxia and radiotherapy alter the stromal compartments and their contribution on tumor cell properties.

The Present Investigation

Methodologies

Murine Glioma Models

In vivo tumor models are animal-based, pre-clinical systems, often conducted in mice to study cancer evolution, drug response and safety. Commonly used model systems include patient derived xenografts (PDX), cell line-derived xenografts (CDX) or genetically engineered mouse models (GEMMs).

Xenograft models are based on injection of cancer cell lines, or implantation of a patient tumor piece into immunocompromised mice. For CDXs, they can either be administered intravenously, subcutaneously or orthotopically. A drawback of this model is that they often not recapitulate the whole tumor diversity seen in other models²⁷⁶. PDXs, on the other hand, are often administered orthotopically and show the same genetic and molecular diversity as found in the original patient tumor²⁷⁷. They often retain their ability to grow invasively, and form metastasis²⁷⁸. PDXs are of particular interest when investigating drug response variation between patients. However, a disadvantage on both above mentioned models are that they are dependent on immunocompromised mice, thereby limiting the impact of the systemic immune system on the tumor biology. In GBM research, CDX or PDXs models are often placed subcutaneously, which restrict their ability to mimic the natural brain microenvironment. Because these models lack a functional BBB, their clinical relevance, particularly for therapies dependent on CNS delivery is difficult to interpret.

Genetic models offer the advantage of studying defined genetic alterations in a fully immunocompetent environment, making them useful for testing therapies that affect the immune microenvironment, such as immunotherapy. They are also particularly important when studying the tumor microenvironment, as the complex interactions between immune, stromal and cancer cells are maintained. However, these tumor models are driven by defined genetic alterations, which may not capture the full diversity and heterogeneity observed in human tumors²⁷⁹. The RCAS/tv-a system used in this thesis, is a transgenic mouse model, that is genetically altered and carries an avian tumor virus A (tv-a) receptor expressed under a promoter of interest²⁸⁰. In our case, the tv-a is present at the *Nestin*-promotor, ensuring that viral infection only occurs in Nestin-expressing cells. This allows to either introduce or silence genes

of interest (such as oncogenes or tumor suppressor genes) in selected cell types at a precise timing. The cell type specific delivery is of particular advantage, since gene expression will not be affected in other cell types as seen in general knock-in and knock-out models. In our research, we have introduced expression of human *PDGFB* (*hPDGFB*), together with silencing of *Trp53* by injecting neonatal mice intracranially with cells expressing RCAS-vectors containing these constructs, which then transform cells positive for *tv-a*²⁸¹⁻²⁸³. Tumors will then start to develop from *de novo* in a native brain tissue microenvironment at the site of injection, allowing to track both initiation, progression and resistance in a model with a fully immunocompetent environment²⁸⁰. Inclusion of red fluorescent protein (*RFP*) reporter in the RCAS-*shTrp53* vector also allows for tracking of transformed cells.

Single Cell RNA sequencing

RNA sequencing is a powerful tool to investigate the global gene expression pattern of a sample. Conventional RNA-sequencing is based on sequencing the whole transcript, generating an average transcript expression of a mixture of cells from a sample, usually called “bulk” sequencing. This data includes transcripts from several cell types present in the sequenced sample, including tumor cells, immune cells and stromal cells. For instance, Verhaak *et al.* used bulk RNA sequencing from TCGA on GBM and initially identified the existence of three GBM subtypes, including proneural, classical and mesenchymal, associated with genetic aberrations in *PDGFRA/IDH1*, *EGFR* and *NF1*⁴⁹. Later, it appeared that *NF1* deficiency was associated with an increased influx macrophages of GBMs⁵⁰. The MES-signature observed in these tumors from bulk RNA sequencing can therefore be influenced by both tumor cell intrinsic characteristics, or tumor environmental factors, such as the presence of tumor associated macrophages that may display MES-like signatures²⁸⁴. The introduction of the scRNA-seq technique allowed researchers to acquire a gene expression profile from each individual cell within a sample or tumor. In comparison to bulk RNA sequencing, this enables highly detailed transcriptomic analysis, which enables scrutinization of the cellular heterogeneity between and within cell types. For its ability to unravel heterogeneity, Nature rewarded it the method of the year 2013²⁸⁵.

The technique itself is based on either poly-A capture, or probe-based assays. Whereas poly-A capture targets all 3'-mRNA molecules, probe-based assays detect targeted transcripts, and allows for addition of custom designed probes. For instance, in our project we wanted to add detection probes for RCAS-vectors, allowing us to detect cells that had *RFP* and *hPDGFB* expression, resulting in an easier distinction between what is malignant and non-malignant cells. Probe-based assays allow for easier detection of low-abundance transcripts, which might be of high relevance for detecting rare subpopulations, for instance glioma cells with stemness features.

Even though scRNA-seq has revolutionized cell biology and provided extremely insightful information on cellular heterogeneity, it still carries some pitfalls. scRNA-seq is based on viable and intact cells obtained from cellular dissociation of the sample specimen, thus loses the spatial context of the cellular architecture and could contain cell type bias during sample preparation. Conclusions based on scRNA-seq involving predicted interactions between cell types should therefore be interpreted with caution, as these cell types might not naturally occur in the same spatial location within tumors.

Spatial Transcriptomics

Spatial transcriptomics works in similar ways as for scRNA-seq, using either 3'-mRNA capture or probe-based assays. Capture from tissue sections occurs on a fixed matrix so that spatial information is retained. This can be utilized by for instance, unique barcodes that are present in capture areas of 2x2 μm -squares, as in 10x Visium HD. Gene expression containing these unique barcode sequences can later be traced back to their spatial location. Spatially resolved transcriptomics further advanced how we understand complex tissues, and was therefore awarded the Method of the Year by Nature 2020²⁸⁶.

Metagene Scores and Interaction

Both transcriptomics methods above, can be used to extensively analyze tumor cell biology. After harmonization of data, it is common to perform cell assignments based on a reference gene set or known markers for each cell type. Typical analysis of cell clusters or between treatment groups include exploring gene set enrichment analysis (GSEA), using well defined gene lists associated with biological pathways, for instance, Hallmark or GO databases used across our studies^{287,288}. Exploring up- or downregulated gene pathways in relation to therapeutic treatment could provide useful information as to which biological targets/pathways may play a role in tumor progression and resistance. More sophisticated analysis has the potential to predict possible intercellular cross-talk, utilizing known receptor-ligand pairs on distinct cell types across experimental conditions. It therefore provides a tool for finding and prioritizing which pathways or targets that significantly differ between experimental groups and may be of interest for downstream analysis.

Paper I: Coevolution of neoplastic and non-neoplastic reactive astrocyte states converges on mesenchymal-like and injury-response programs during murine glioblastoma progression and post-radiotherapy recurrence

Tumor recurrence and therapy resistance remain the limiting factors of GBM prognosis. Recent decades have focused on unraveling the role of the TME in disease progression. Stress factors, such as chemotherapy and radiotherapy, are not confined to tumor cells alone, but most likely affect the phenotypic behavior of a spectrum of cells within the GBM microenvironment. The underlying mechanisms of how standard of care therapy influences the regrowth of treatment resistant tumor cells and modulates the microenvironment therefore needs to be further addressed. In this study, we aimed to explore how stress from radiotherapy drives phenotypic alterations in both cancerous and non-cancerous cell populations within GBM.

Major findings

In this study, we utilized ScRNA-seq from healthy brain, primary- and post-radiotherapy recurrent- tumors from RCAS-hPDGFB and shTp53-induced murine GBM to explore how neoplastic and non-neoplastic compartments alter in tumor progression (illustration of study design in Figure 6). To detect malignant cells, we used customized probes against human platelet growth factor B (*hPDGFB*) and *RFP* on the RCAS-vectors. Overall, we detected 10 malignant states and 13 non-neoplastic cell types in our dataset. Non-neoplastic cells were clustered followed by annotation using the Allen MapMyCells tool. All major glial and stromal cell types typically found in brain tissue were identified, such as oligodendrocytes, astrocytes, microglia, neurons, fibroblasts, choroid and endothelial cells. We observed substantial shifts in cellular populations in post-radiotherapy recurrence tumors, including a transition from microglia-dominated primary tumors to bone marrow derived macrophages in recurrences, as concurrent with previous literature²²¹. In addition to this myeloid reorganization, immunogenic populations such as dendritic cells, neutrophils and lymphoid (NK/T/B) cells were markedly elevated in tumor tissue relative to healthy brain, with the strongest enrichment occurring in recurrent samples.

Ravi *et al.* utilized spatial transcriptomics to reveal that human GBM is organized into distinct spatial neighborhoods, including neurodevelopmental zones as well as hypoxia-associated mesenchymal and inflammation-associated niches, where immune-reactive signatures align with a MES-AC-like hybrid state⁸¹. Consistent with this, studies by Neftel *et al.* and Nicklasson *et al.* support the existence of a

distinct astrocyte-mesenchymal-like tumor cell state^{63,80}. In our model, post-radiotherapy recurrences exhibited a reconfiguration of the microenvironment and tumor cell states. Neoplastic cells shifted toward injury-response, ACR/MES-like programs marked by ECM-remodeling, while reactive astrocytes emerged as the principle non-malignant interaction partner of ACR-like tumor cells. This coupling was reinforced by spatial concordance between the Richards Injury-Response program, the Neftel MES1 signature and our neoplastic ACR state. In line with prior work, reactive astrocyte signatures were even more pronounced in recurrent GBMs compared to primary lesions¹⁵⁰. Intriguingly, reactive astrocytes displayed transcriptional features spanning both neurotoxic “A1” and neuroprotective “A2” states, emphasizing that astrocyte reactivity is not binary but instead represent a diverse, context-dependent continuum. Their interactions were mostly based on adhesion molecules and matrix-organization pathways, consistent with fibrosis-like scarring after radiation. Adding to this, Xie *et al.* analyzed matched and primary recurrent human GBM samples, and identified a stem cell-like cluster with high *AQP4* and *GFAP* expression, markers of astrocytic lineage, enriched shortly after chemotherapy, and more abundant in recurrence. Uniquely, this cluster alone could re-introduce tumors whereas the remaining failed, suggesting a pivotal role in tumor expansion and resistance²⁸⁹. Similarly, we observed a significant increase of GFAP⁺ tumor cells at 72 hours after radiotherapy. Comparing primary and recurrent GBMs, we uncovered secreted (EGF, Wnt and Gas) and ECM (Collagen, Tenascin and Thbs) signaling pathways between astrocytes and tumor cells that may fuel this. Moreover, a study by Marallano *et al.*, reported genotype-specific responses within hypoxic niches, where IDH-mutant tumors enrich AC-like states, whereas IDH-wt tumors favored MES-like states, potentially reflecting stronger injury-response programs in higher grade tumors²⁹⁰. Together, these findings align GBM along a developmental-to-injury continuum and support a model of tumor-stroma coevolution under therapeutic and microenvironmental stress, in which reactive astrocytes act as central architects to potentially drive the recurrent niche. This is further supported by resection-models, showing that trauma induced by resections also actively contribute to a peritumoral reactive astrocyte profile and tumor recurrence¹⁵¹. This suggests that residual cells exposed to therapy-induced stress can drive tumor regrowth, helping explain why recurrences typically arise near the original tumor site. By coordinating ECM dynamics and niche signaling, reactive astrocytes may therefore be one of the key drivers of an ACR/MES-like phenotypes, associated with treatment resistance and regrowth after radiotherapy.

Collectively, our results from this paper points toward a stress-driven coevolution of tumor and stromal cell compartments that shapes the cellular and molecular features of recurrent disease where neoplastic cells adopt reactive-astrocyte-like, ECM-remodeling programs (ACR/MES-like), whereas primary tumors retain NPC/OPC-like states associated with developmental signatures and less environmental stress. Together, this integrated view, highlights opportunities to disrupt astrocyte-tumor crosstalk as part of recurrence directed therapy.

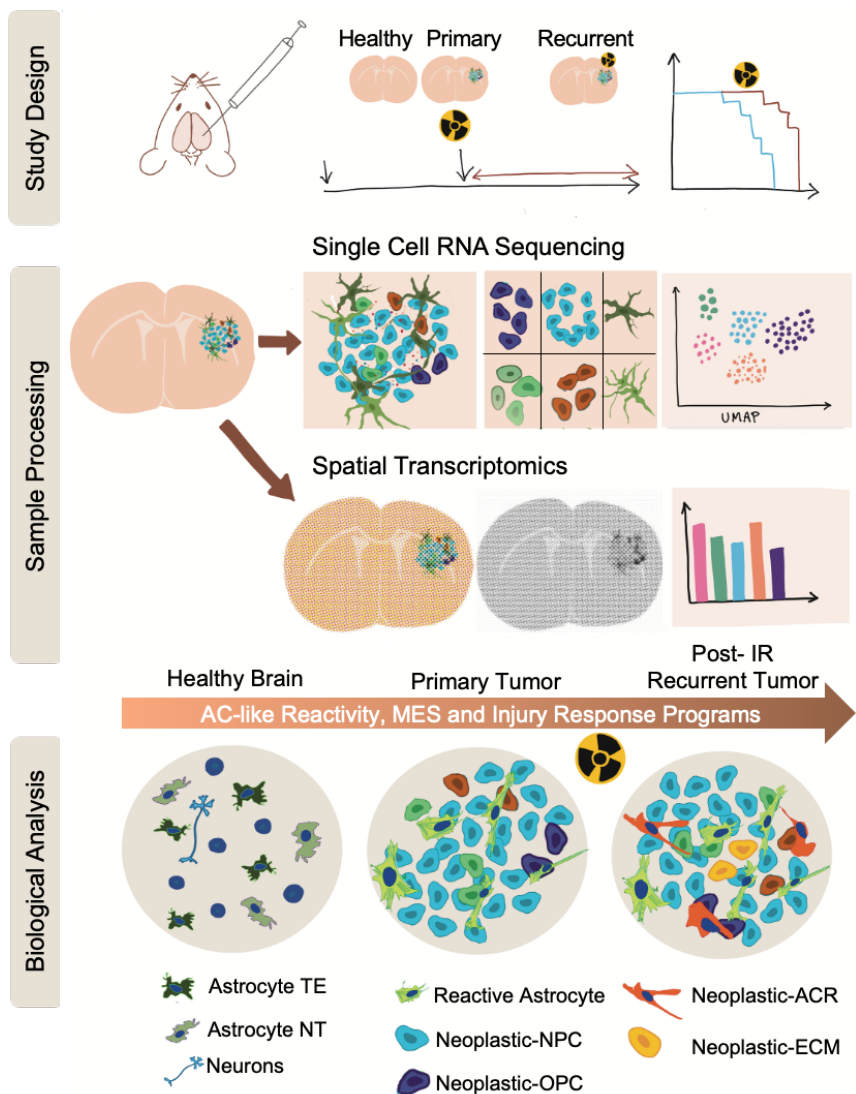


Figure 6. Schematic illustration of the workflow in Paper I.

Paper II: Phenotypic Screening Identifies Flunarizine as an Inhibitor of Radiotherapy-Induced Astrocyte Reactivity with Therapeutic Potential in Glioblastoma

In paper II we explored drug repurposing for targeting astrocyte reactivity in treatment of glioblastoma. Following up on Berg *et al.*¹⁴⁰ who identified irradiation-induced astrocyte reactivity as a driver of tumor aggressiveness, our aim with this project was to determine whether pharmacological compounds could selectively target the reactive astrocyte-mediated tumor progression that arise following radiotherapy.

Major findings

Astrocyte reactivity is a crucial biological response that protects our brain from tissue damage, but its dysregulation contributes to diverse CNS diseases and brain tumors²⁹¹. To quantify this phenotype, we developed an automated image-based assay in primary human astrocytes that tracks morphology (cell and nuclear features) together with established markers, enabling high throughput unbiased detection of IR-induced reactivity. We found that the irradiation-induced effects corresponded well with features of astrocyte reactivity described in literature, like increased cellular size, as well as increased GFAP and vimentin expression. Using this platform, we screened FDA-approved compound libraries with the aim to identify compounds able to inhibit IR-induced reactive phenotype and identified flunarizine, a well-tolerated anti-migraine drug as a top hit that consistently reverted IR-induced astrocyte features without toxicity. Transcriptomics showed IR activates TP53, TGF- β , EGFR, and ECM programs, whereas flunarizine dampened TGF- β signaling and rescued reactivity-linked ECM components such as Tenascin C (Tnc) or TGM2. As concurrent with previous literature, we found that that reactive astrocytes contribute to fibrotic scar formation after injury, as IR increase type I Collagen deposition and Tnc expression, both associated with poor survival in GBM.

In vivo, flunarizine suppressed astrocyte reactivity, as measured by GFAP, across GBM and brain-metastasis models and, when combined with radiotherapy, prolonged survival of mice. This effect seems mainly to be because of lowered Tenascin C and Collagen I deposition, as the astrocyte density were similar in primary and recurrent tumors, further suggesting that their reactivity rather than only abundance is important⁷⁶. In line with our previous observations in Paper I, recurrent tumors showed enrichment of Tenascin C and Collagen signaling originating from reactive astrocytes toward neoplastic cells. Consistently, recombinant Tenascin C enhanced colony formation in PDGFB-driven primary

glioma cultures. Our results align well with previous literature, where CSF-1R inhibition lower macrophage infiltration and extends survival, yet recurrences form within protective fibrotic scars driven by TGF- β ECM-remodeling, and that blocking this glial-scar formation together with CSF-1R inhibition markedly improves long-term survival^{247,292}. Together, these results validate astrocyte reactivity as a pro-tumorigenic driver, supporting tumor regrowth after radiotherapy, and position flunarizine as a possible drug repurpose candidate to dampen IR-induced astrogliosis and matrix remodeling, offering a practical route to enhance radiotherapy across GBM and brain metastases.

Paper III: Hypoxia-Induced Complement Component 3 Promotes Aggressive Tumor Growth in the Glioblastoma Microenvironment

Hypoxia and pseudopalisading necrosis are a well-established hallmarks of GBM. Although tumor-intrinsic hypoxic responses have been extensively studied, much less is known about how stromal signaling within hypoxic niches contributes to disease progression. In Papers I and II, we showed that the TME adapts to radiotherapy by activating injury-response programs and remodels the extracellular matrix in a pro-tumorigenic manner. In these contexts, astrocytes emerged as key drivers of tumor aggressiveness and survival under stress. Building on this, the aim of this project was to investigate whether additional forms of cellular stress, such as hypoxia, also shape tumor progression, radio-resistance or immune evasion of GBM.

Interestingly, studies of neurodegenerative diseases have identified complement component C3 as a prominent marker of astrocyte reactivity. However, its presence and functional relevance in brain tumors remain unclear. This observation inspired us to examine whether complement proteins are expressed in tumor associated astrocytes, and their roles in GBM biology.

Major findings

In paper III, we identified a strong association between hypoxia and local complement signaling using bulk RNA-sequencing (TCGA), scRNA-seq²⁹³, and spatial transcriptomics⁸¹ from human GBM datasets. In our transgenic mouse model, we saw that C3 was specifically enriched in hypoxic and perinecrotic tumor regions, indicating that C3 is not uniformly distributed but instead displays distinct spatial patterns within tumors. While this correlation is likely driven in part by tumor-associated macrophages and microglia (cell types that are enriched in

hypoxic niches and major contributors to complement expression), we also demonstrated through oxygen-level titration experiments that *C3* and *C3AR1* are directly upregulated by hypoxia in multiple cell populations. These findings extend earlier work connecting hypoxia to complement signaling, including studies showing increased hypoxic responses in tumors with complement-pathway mutations and hypoxic regulation of complement-related genes across various cancer models^{294,295}.

To examine the functional consequences of this, we next tested how presence of C3 in culture medium affected tumor cell behavior. Although initial observations suggested that C3 might exert a protective role under stressful conditions, consistent with reports in other cell types, these findings were highly variable and did not allow for any firm conclusions. Such variability may reflect several technical and biological factors, such as the absence of other cofactors required for C3 conversion into its active form, potential differences between extracellular or intracellular functions, or variability in receptor expression across cell lines.

When we shifted focus to complement receptors and analyzed TCGA data, we found that *C3AR1* is more highly expressed in GBM than in any other common cancer type, and with expression levels correlating with poorer overall survival. ScRNA-seq further showed that *C3AR1* is expressed predominantly by microglia and macrophages, with lower expression detectable in a subset of malignant cells. In the physiological brain, C3a/C3aR signaling plays key roles in neural development, hypoxic-ischemic injury responses, and regulation of BBB and vascular integrity, all pathways that could have implications in glioma biology²⁹⁶⁻²⁹⁹. Moreover, this pathway has been linked to stemness, migration, proliferation and angiogenesis across several cancers³⁰⁰. Given its high expression in gliomas, we hypothesized that the C3a/C3aR axis could be a viable therapeutic target in GBM, and indeed, combining radiotherapy with C3aR inhibition (SB290157) extended survival of glioma-bearing mice, aligning with recent findings in pancreatic cancer models³⁰¹.

The observed survival benefit appears to be driven by alterations in the immune microenvironment, as treated tumors contained fewer M2-like macrophages in perivascular niches, as illustrated in figure 7. Reducing immunosuppressive macrophages in GBM have previously shown to be beneficial by targeting the CSF-1/CSF-1R-axis, which pro-longed survival in glioma models^{292,302}. Likewise, blockage of C3aR1 expressing macrophages through anti-C3a therapy has reduced tumor growth in other malignancies. *In vitro* exposure of microglia or primary murine macrophages to C3a induced M2 polarization, reinforcing this link. Another study by Zhang *et al.* published around the same time as ours, independently showed M2-like polarization by tumor associated macrophages in response to C3a/C3aR-signaling, which was abrupted in presence of a different C3aR inhibitor (JR14a)³⁰³. This study also linked the C3a/C3aR axis to glioma phenotypes, showing that C3a promoted MES transition of PN glioma stem cells via both M2-macrophages, as

well as direct binding of C3aR on glioma cells, and disrupting this axis led to impaired growth of glioma cells.

Our results indicate that hypoxia-driven C3a/C3aR signaling, may help establish the M2-dominant immunosuppressive microenvironment typically found in hypoxic or perivascular tumor regions and can help fuel the mesenchymal subtype of GBM often associated with those niches. Therapeutically targeting this axis could therefore be beneficial in GBM.

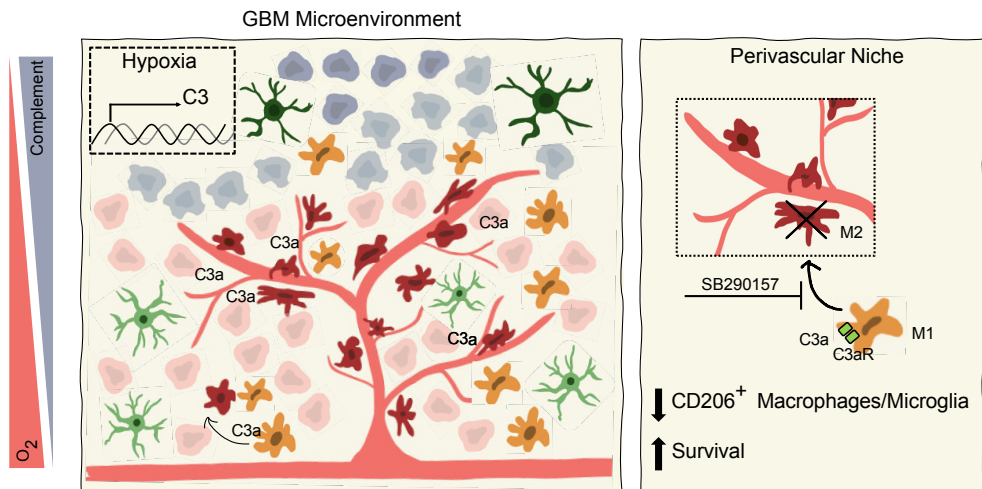


Figure 7. Graphical summary for paper III.

Paper IV: Hypoxia-Induced Reactivity of Tumor-Associated Astrocytes Affects Glioma Cell Properties

The ways in which astrocytes, a major stromal cell type in GBM, respond to hypoxia, and how hypoxic astrocytes influence glioma cells are still not fully defined. Insights from ischemic injury models show that reduced oxygen tension drives astrocyte into a reactive, functionally altered, and neuroprotective state. The aim of this project was to investigate how astrocytes adopt to hypoxia, and how stromal cells in local tumor niches affect glioma cell properties.

Major findings

Although the effects of hypoxia on tumor cells are known to drive invasion, migration and angiogenesis, it is still left to explore how hypoxia affect stromal cell populations. In this study, we demonstrated that primary human astrocytes

underwent robust hypoxia-driven reactivity, which was dominated by HIF-2 α under intermediate/severe hypoxia (1-0.1% O₂). Hypoxia triggered distinct temporal and morphological changes (Figure 8), with Vimentin expression increasing at 24 hours after RT but normalizing by 72 hours. In contrast, cell hypertrophy, assessed by cell area did not emerge until 72 hours. These dynamics underscore the heterogeneity of astrogliosis, as some reactive features are transient or reversible. Furthermore, together with Paper II, this highlights the need to objectively identify astrocyte reactivity using a broad set of markers and features, given that it is strongly context dependent.

Consistent with activation of canonical hypoxia pathways, classical HIF target genes, including *PGK1*, *NDRG1*, *CA9*, *BHLHE40*, *SLC2A1*, *VEGFA*, *BNIP* and *MXII* were upregulated as oxygen availability decreased. Under prolonged severe hypoxia, astrocytes preferentially stabilized HIF-2 α , suggesting that HIF-responses may be temporally regulated in astrocyte reactivity^{304,305}. Notably, Johansson *et al.* reported that CD44 promotes HIF-2 α stabilization in glioma cells within hypoxic and perivascular niches, thereby enhancing stem-like properties.

CD44 is a well-known marker of reactive astrocytes³⁰⁶. Although not examined in this study, it is tempting to speculate that CD44 could similarly influence HIF-2 α stabilization in reactive astrocytes in perivascular and hypoxic niches. This possibility is particularly relevant given our findings in Paper I, where tumor-associated astrocytes showed high hypoxic signature scores. It remains an open question whether these astrocytes also exhibit pseudo-hypoxic phenotype, as they reside both in avascular tumor regions and in perivascular zones near tumor borders. Furthermore, in a breast cancer model HIF-2 α was shown to protect tumor cells and promote stemness in hypoxic stress by upregulating SOD2, thereby reducing mitochondrial ROS³⁰⁷. Similarly, in obesity models HIF-2 α protects β -cells from metabolic stress by SOD2 expression, and knockout of HIF-2 α resulted in glucose intolerance, and β -cell dysfunction³⁰⁸. This indicates a protective role of HIF-2 α in buffering mitochondrial dysfunction caused by environmental or metabolic stress, in part through the upregulation of antioxidant pathways.

Hypoxic astrocytes also exhibited enhanced secretion of hypoxia-associated cytokines such as TGF- β 1, VEGF-A, angiogenin, IL-3, and IL-1 α , associated with regulation of fibrosis, angiogenesis and immunomodulatory pathways, consistent with findings from paper I-III. Interestingly, IL-1 α is a potent pro-inflammatory cytokine that has also been shown on surface of senescent cells, further contributing to the SASP phenotype^{134,309}.

Functionally, glioma cells cultured on extracellular matrix produced by hypoxic astrocytes displayed increased colony-forming capability and enhanced stem-like features, supporting the idea that astrocytes within hypoxic tumor niches, contribute to glioma stemness via the extracellular matrix. Overall, we found that astrocytes

respond to tumor hypoxia, by becoming reactive, similarly to their response to radiotherapy, and that hypoxia-reactive astrocytes actively shape a microenvironment that promotes glioma aggressiveness.

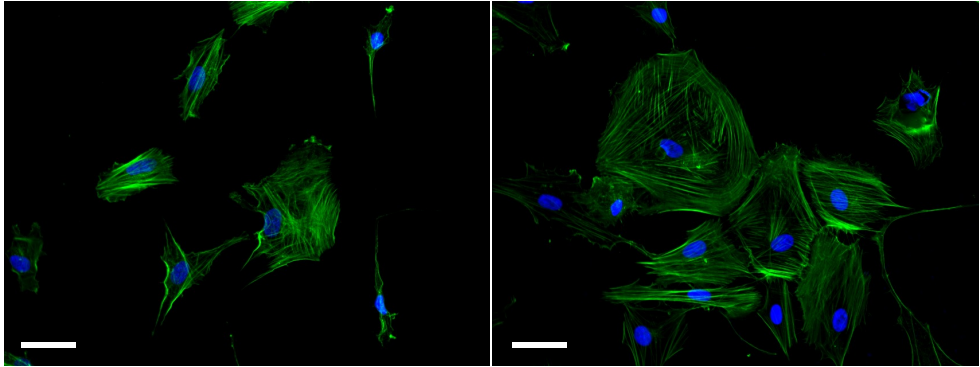


Figure 8. Primary human astrocytes grown in 21% (left) versus 0.1% O₂ (right) displayed with nuclear stain DAPI (blue) and cytoskeletal marker phalloidin (green). Scale bar 50 μm.

Summary and future perspectives

GBM tumor recurrence typically appears within a 2-cm radius of the primary lesion, an area that has already received high dose radiotherapy treatment. It therefore seems reasonable to debate that future therapeutic strategies must either eliminate treatment-resistance tumor clones, restrict pro-tumorigenic stromal factors, or enhance immune-mediated clearance of residual tumor cells. Across the projects included in this thesis, we have collectively investigated how stromal and immune compartments, particularly astrocytes and macrophages/microglia together with tumor cells themselves respond to microenvironmental stressors such as hypoxia and radiotherapy. Throughout four complementary studies, we show that injury, inflammation, hypoxic signaling, and astrocyte-mesenchymal (AC/MES)-like reactivity emerge as interconnected drivers of gliomagenesis and radioresistant tumor evolution.

Although we used a transgenic mouse model, this system allowed us to study tumor evolution from a consistent genetic background without sampling biases that can complicate human GBM biopsies. Limitations of our studies are present due to potential biases in cell type populations that can arise during enzyme-based tissue dissociation, and may explain why certain cell types, such as astrocytes and neurons often are underrepresented in scRNA-seq datasets. To overcome this, new technologies have emerged and single-nuclear RNA seq are now possible from fixed FFPE tissue, thereby overcoming the isolation difficulties of several stromal cells. Nevertheless, our scRNA-seq data convincingly highlight reactive- and injury driven pathways as major regulators of astrocyte-mesenchymal transition and microenvironmental remodeling. Across all studies, TGF- β and TNF- α emerged as common central mediators, where TGF- β orchestrate ECM remodeling and injury responses, and TNF- α promotes inflammation. In collaborative work with Tuyuz *et al.* we further linked TNF- α /NF- κ B/STAT3 signaling to tumor aggressiveness, regulated by the complement inhibitor CSMD1³¹⁰. In Paper III, cells with high C3 expression had an elevated TNF- α signaling pathway, suggesting that these two interconnected innate and inflammatory pathways act together in tumor inflammation. Additionally, TGF- β links complement activation with angiogenesis via regulation of VEGF, C3, and C3aR expression in tumor associated macrophages³¹¹. Furthermore, in collaboration with Smolag *et al.* we demonstrated that Factor H, a complement inhibitor that limits excessive alternative pathway activation, has immunomodulatory properties. By binding to ICOS on T regulatory

cells (Tregs), FH enhances Treg viability and suppressive function by increasing IL-10 and TGF- β secretion and inhibiting effector T cell proliferation³¹². These findings further demonstrate the paradox and complexity of the GBM microenvironment, where TGF- β either via complement activation, or complement regulatory proteins, provides a link between immunomodulatory systems and glial scar formation.

This duality reflects a central paradox of radiotherapy in GBM, as while radiotherapy kills many tumor cells, it simultaneously generates a reactive, protective niche that supports recurrence. Overall, our findings show that as tumors evolve, they increasingly engage interconnected reactive, injury-driven, and inflammatory pathways that reinforce each other to drive progression. This supports the long-standing concept that “*cancer is a wound that never heals*”.

Future directions

This thesis contributes to the ongoing field to understand how stromal and tumor cells transition and adopt between states. As extensively demonstrated, GBM has profound plasticity in response to treatment and tumor niches. Therapeutic targets will therefore need to be investigated thoroughly in a temporal resolution, as the same therapeutic may yield different outcomes depending on the time of administration. A few promising therapies are related to targeting the SASP-mediated pathway or other stromal components that frequently occur in response to radiotherapy. Other targets may include differentiation or transition medicines, that forces cellular transition to a state more susceptible to therapy. To demonstrate a beautiful example of this, is the usage of all-trans-retinoic acid (ATRA) with arsenic trioxide therapy in acute promyelocytic leukemia, which is also now being tested in GBM^{313,314}.

“Tumor cells that once were immature and deadly in my marrow, woke and poured into the river of my blood. The bone was a midnight freeway with exits to everywhere. Lanes of light in fevered rush. Like a magnolia tree opening all at once. I felt their elegance and ache, pushing against the corridors of my bones, a blossom of all their ugly clones.”

Acknowledgements

I would like to express my sincere gratitude to all those who helped me complete this PhD journey.

First and foremost, my main supervisor, **Alexander**. I would like to say, *a very strong thank you*, for being my supervisor these past years, and all that it included. You and your expertise have taught me invaluable knowledge and shaped me as a scientist. Thank you for teaching me about the complexity of the tumor microenvironment, and how to be excited about continuing to explore it. You have supported me in forming my own ideas and guided me in how to follow them through. And every time I was in doubt, you taught me the most important scientific method; the more you *fiddle* around, the more you figure out. Being a student in your lab has been a great part of my life, that developed me both scientifically, and personally. And with that said, lastly, thank you for being my involuntary therapist for the past five years.

Elinn, thank you for being my co-supervisor and life-coach. I have valued every moment sharing an office with you, from having weekly positive Tuesday affirmations to our unsuitable ‘after-five a clock’ conversations. You have made my PhD experience more knowledgeable, supportive, creative and most important of all – giggly. I must say, I particularly enjoy the parts when we do lab work together (like an enzyme and substrate – we work better together). I hope we will continue our “jaja”-conversations until we are in the appropriate age of actually having them.

Pauline, thank you for sharing your expertise with me throughout the years, and for all our times spent together inside and outside of the lab. Thank you for introducing ‘real’ raclette to me! And I strongly value the unicorn that you made for me, and still have it by my bedside table (I imagine it brings me luck). **Karolina**, we now celebrate 10 years together! I cannot believe how quickly time has passed. There are so many moments to say thank you for (apart from stealing my blood for experiments). I have enjoyed all our philosophical conversations, M’ with olive nights (and finally we got that tattoo!), and sky views on mountain tops together in Vancouver, Norway and Schweiz or from skyscrapers in New York. I like how we can share the same excitement about things in life (especially food), and in science. I look forward to continuing exploring life with you. **David**, thank you for all coffee and wordle breaks (even though I sometimes had to force you), and for all your heavy work in our shared projects. It has been highly valued. **Maria**, I would just like to say how much I have appreciated working with you – it has been fun to tackle

obstacles together, such as embedding those extremely tiny organoids. You always come with good advice and offer a helping hand. **Suey** and **Somdatta**, it has been fun to get some new additions to the lab. You've brought a new positive vibe, and I am excited to get to know you both more! To previous members of the lab, **Christina**, **Dimitra**, **Vasiliki** and **Tracy**, it was fun to start and being in the lab when you were around. You created a welcoming atmosphere and were always up for a challenge, especially if it involved escape rooms. Thanks to my previous students, **Julia** and **Christina**, who helped me greatly in my projects.

Göran, thank you for being my co-supervisor. I am grateful for the collaboration we have had between our groups. Particularly thank you to **Bengt**, **Katja**, and **Kamila** with all your patience and help practically (and mentally) while setting up new techniques. It has been really fun getting to know you **Jacob** as well, and getting a partner in crime for bingo evenings. A special thank you to our other collaborators and **co-authors** that have helped me with providing guidance, feedback and help along this thesis. **Anna**, my first research experience was in your lab, it provided me with skills and knowledge that have been extremely useful for me for the rest of my scientific journey. Somehow, complement has continued to sneak into my work. **Kristian**, it has been very fun to work with you and your group. I have enjoyed all our shared work with the tumor microenvironment and activities, involving fencing, boule, and ping-pong. Especially thanks to **Sebastian** to whom I shared joy and anxiety about Visium, and **Jonas** for all your bioinformatical skills and lunchroom conversations of projects, your help has been highly valued. And **Sophie**, for knowing exactly the right timing of support-snacks (Parmesan & Truffle chips). **Crister**, you have played a central role in all our work in this thesis, by always offering help, availability and knowledge when it comes to radiotherapy.

Jessica and **Lexi**, I loved that we had each other during this journey and that we all were students with pensioner souls. Although the years across our PhD came with different obstacles, battling them together has made it easier. There is a beauty in being able to joke about hard times together. Crafting, Magic Mike, and pizza evenings definitely helped also.

I would also like to thank **everyone at TCR** including all past and current members as well as fellow PhD students, **Klaudia**, **James**, **David**, **Natalie**, **Jacob**, **Lexi**, **Jessica**, **Sandra**, **Katarzyna**, **Clara**, **Dora** and **Emma L** for creating the vibrant and welcoming atmosphere that we have at Medicon Village. I particularly fancied the times in the CARES course, and thank you **Johanna** and **Håkan** for making that happen so successfully as it was. I will for sure miss the spontaneous talks and smiles from you all, **Margareta**, **Sophie**, **Daniel**, **Yvonne**, **Kristina**, **Javan** and **Karin** in the corridors.

I would also like to take the opportunity to acknowledge some friends and family, for all their support during these last few years.

Johan, Ben, Tamae, Kristofer, Tina and Anette, thank you for all our years together in the company, bike rides to saltimporten, and good old times at the Pathology building in Malmö, and for how you, and **Leif**, encouraged me to pursue a PhD in the first place. **Sabine**, thank you for making my hospital stay so much more enjoyable by kidnapping me from my room and racing in office chairs around the hospital culvert. It was the most fun I had in a while. *RIP dino*, **Carro, Emma, Frida and Marie**, we started our time in Lund together forever ago. With you, I've had the best time of my life. We've lived together, had baby kittens (and now some real babies), shared enthusiasm for music (Carro, my concert-Queen), and had limbo/vacuuming evenings with Léon in the speakers. I look forward to our many years ahead. **Josse, Kostas, Karolina, Gregi and Ben**, thank you for making my life outside of the lab so much richer. I've had so many fun experiences with you, including brunches, Bond nights and memorable holidays. **Robin, Julia, Schiele, JP, Michaela, Tellan, Maja and Piffe**, thank you for adding to my life with all our crazy adventures. You have been there throughout the good and the worst. I love you for that. **Alexandra, Elinn and Anna** our evenings always come exactly when needed! **Kylyn**, even if we most of the times have large oceans between us, I always feel like I have you by my side. Thank you for being the most amazing friend.

Till min familj, **Mamma och Pappa**, som alltid ställt upp och stöttat mig. Tack för att ni körde halva Skåne runt för att hitta mitt gamla favoritgosedjur när jag blev sjuk. Men också för att där alltid finns grillad mat från 1 Januari-31 December hemma hos er efter en lång vecka i labbet. **Robin**, du är inte bara min bror utan även den bästa vän jag någonsin hade kunnat önska mig. **Julia**, jag är glad att vår familj har blivit berikad av dig, du är som den icke-biologiska (*nästan*) system som jag slutligen fick. Det har varit roligt att få dela entusiasm för vetenskap med dig. Ni och **Moster Gun & Boje** med **kusinerna** har varit en otrolig support! Kram till er **farmor, farfar, mormor och morfar** (i tanken varje dag). Slutligen **Ben (Bennyboo)**, tack för att du har varit vid min sida genom allt, och den stöttning och peppande du gett mig varje dag under de senaste åren.

Jag skulle vilja ge ett extra stort tack till Hematologiavdelningen i Lund, för utan er hade jag inte kunnat göra färdigt min doktorsavhandling♥. **Cecilia och Emelie**, ni har varit klippor under den värsta perioden av mitt liv, och fick mig att skratta varje dag. Jag har uppskattat alla våra foodie och katt-konversationer. **Lars**, jag känner en enorm tacksamhet till dig. Hur du alltid får mig att känna mig inkluderad i min egen vård, och tar dig tid att sitta ned och diskutera allt från bieffekter, existentiella frågor och vetenskapen bakom vad som sker i kroppen. Men också för alla tips inför hiking-resor i Norge! **My**, tack för att du hjälpte mig hitta vägen tillbaka igen.

Ett stort hjärta till alla nya goa vänner jag fått lära känna de senaste två åren genom WAYA studien, Lydiagården och Unga Vuxna med Cancer. Allt har känts lite mindre ensamt att vara i bubblan **tillsammans med er**.

Finally, I would like to thank all the funders that made this work possible; European Union (ERC), the Swedish Cancer Society, the Swedish Research Council, the Swedish Childhood Cancer Fund, the Ollie & Olof Ericsson Foundation, and the Crafoord Foundation, Mrs. Berta Kamprad's Cancer Foundation, Royal Physiographical Society of Lund and the L2CancerBridge program at the CREATE Health Cancer Center.

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