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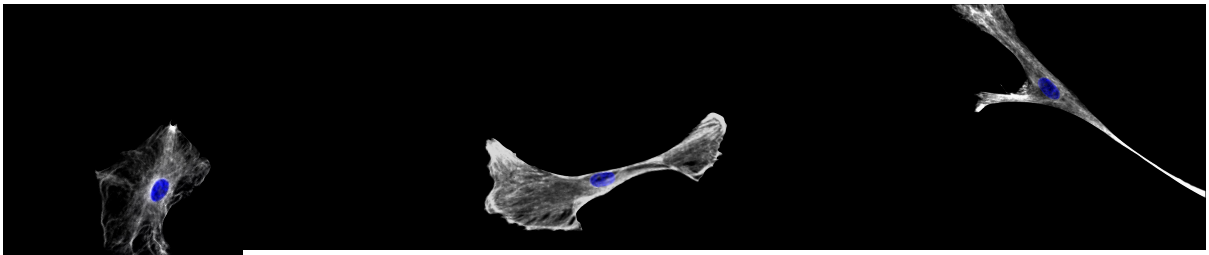

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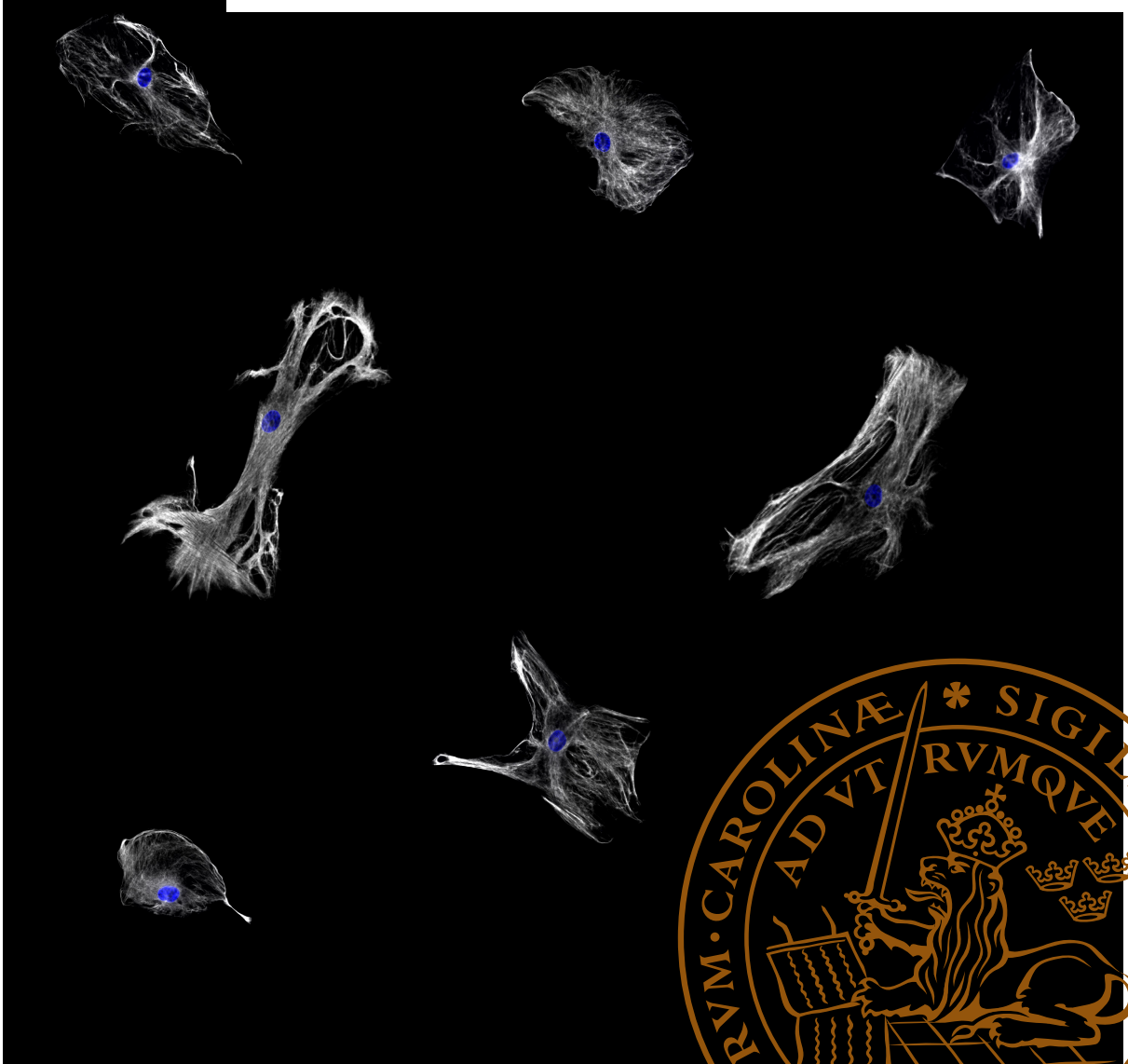

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LUND
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Stromal and tumor cell responses to hypoxia and treatment within the glioma microenvironment

Abstract

Glioblastoma is the most aggressive primary brain tumor in adults. Despite treatment, tumors invariably recur, and the recurring tumor is resistant to therapies. New approaches are needed for the successful treatment of glioblastoma patients.

Tumors are not simply a compilation of molecularly and phenotypically identical neoplastic cells. Instead, the tumor-associated stroma is instrumental in supporting tumor growth. Moreover, the cancer cells themselves are highly plastic, with some of the cells exhibiting stem-like phenotypes. Cancer stemness is linked to more aggressive disease, recurrence, and worse patient outcomes in several cancers. Hypoxic signaling, mediated by the HIF transcription factors, is a cornerstone in the maintenance of cancer stemness in glioblastoma and other cancers.

The aim of this thesis was to evaluate how microenvironmental cues affect the interactions between the tumor microenvironment and glioma stem-like cells in glioblastoma. We addressed how treatments and hypoxia affect tumor-associated astrocytes in ways that consequently alter glioma cell properties, and how hypoxia and pseudo-hypoxia are involved in stemness maintenance in glioblastoma. For this work, we used genetically engineered mouse models of glioma, primary stromal and glioma cell lines, classical glioblastoma cell lines, and organotypic slice cultures. We evaluated cell stemness by using multiple functional assays in combination with stem cell marker expression analysis.

In papers I and II, we investigated the response of astrocytes to extrinsic factors of the microenvironment, namely radiation and temozolomide treatment, and to intrinsic factors of the microenvironment, namely intermediate and severe hypoxia. Astrocytes became reactive in response to these cues and produced extracellular matrix that altered glioma cell properties, including stemness.

In papers III and IV, we investigated the role of hypoxia and pseudo-hypoxia in the maintenance of aggressive glioma phenotypes. We showed that the generation of the cleaved form of the cell surface glycoprotein CD44 leads to the stabilization of the HIFs in the perivascular and hypoxic glioma niche, leading to increased hypoxic signaling and glioma cell stemness. Moreover, we showed that $p75^{NTR}$ signaling is involved in the activation of the hypoxic signaling pathway and is also regulating glioma cell stemness and migration in hypoxia.

All in all, this thesis elucidated some aspects of the glioblastoma microenvironment, namely irradiated and hypoxic tumor-associated astrocytes, and the CD44 and $p75^{NTR}$ signaling, that can lead to the development of new targeted therapeutic strategies.

Key words: Cancer, glioblastoma, tumor microenvironment, reactive astrocytes, hypoxia, CD44, $p75^{NTR}$

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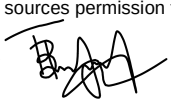
Signature  Date 2021-09-22

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Στην Καλλιοπίτσα

"You cannot stay on the summit forever; you have to come down again. So why bother in the first place? Just this: What is above knows what is below, but what is below does not know what is above. One climbs, one sees. One descends, one sees no longer, but one has seen. There is an art of conducting oneself in the lower regions by the memory of what one saw higher up. When one can no longer see, one can at least still know."
— René Daumal

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List of original papers

This thesis is based on the following papers:

I. The irradiated brain microenvironment supports glioma stemness and survival via astrocyte-derived Transglutaminase 2.

   Tracy J. Berg, Carolina Marques, **Vasiliki Pantazopoulou**, Elinn Johansson, Kristoffer von Stedingk, David Lindgren, Pauline Jeannot, Elin J. Pietras, Tobias Bergström, Fredrik J. Swartling, Valeria Governa, Johan Bengzon, Mattias Belting, Håkan Axelson, Massimo Squatrito, Alexander Pietras

   Cancer Research. 2021 Apr 15;81(8):2101-15

II. Hypoxia-induced reactivity of tumor-associated astrocytes affects glioma cell properties.

   Vasiliki Pantazopoulou, Pauline Jeannot, Rebecca Rosberg, Tracy J. Berg, Alexander Pietras

   Cells. 2021 Mar 10;10(3):613

III. CD44 interacts with HIF-2alpha to modulate the hypoxic phenotype of perinecrotic and perivascular glioma cells.

   Elinn Johansson, Elisa S. Grassi, **Vasiliki Pantazopoulou**, Bei Tong, David Lindgren, Tracy J. Berg, Elin J. Pietras, Håkan Axelsson, Alexander Pietras

   Cell reports. 2017 Aug 15;20(7):1641-53

IV. The p75 neurotrophin receptor enhances HIF-dependent signaling in glioma.

   Bei Tong*, **Vasiliki Pantazopoulou***, Elinn Johansson, Alexander Pietras

   Experimental cell research. 2018 Oct 1;371(1):122-9

The star (*) indicates equal contribution.

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Papers not included in the thesis

1. Niche-derived soluble DLK1 promotes glioma growth.

   Elisa S. Grassi, Pauline Jeannot, **Vasiliki Pantazopoulou**, Tracy J. Berg, Alexander Pietras

   Neoplasia. 2020 Dec 1;22(12):689-701

2. Hypoxia-induced release, nuclear translocation, and signaling activity of a DLK1 intracellular fragment in glioma.

   Elisa S. Grassi, **Vasiliki Pantazopoulou**, Alexander Pietras

   Oncogene. 2020 Mar 24;39(20):4028-44

Abbreviations

ABCG2	Broad substrate specificity ATP-binding cassette transporter
ADAM	A disintegrin and metalloproteinase
ADM	Adrenomedullin
Akt	Protein kinase B
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATM	Ataxia telangiectasia mutated
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
bHLH	Basic helix-loop-helix
BHLH340	Basic helix-loop-helix family member E40
C/EBPβ	CCAAT/enhancer-binding protein beta
CA9	Carbonic anhydrase 9
CAD	C-terminal activation domain
CBP	CREB-binding protein
CCNG2	Cyclin G2
ccRCC	Clear cell renal cell carcinoma
CD44v	CD44 variant isoform
CO_2	Carbon dioxide
CODDD	C-terminal oxygen dependent degradation domain
CSC	Cancer stem-like cells
CSF-1	Colony-stimulating factor 1
CSF-1R	Colony-stimulating factor 1 receptor
CTF	Carboxyterminal fragment
Cx43	C-tail of connexin 43
CXCL12	C-X-C motif chemokine ligand 12 (also known as SDF1)
CXCR4	C-X-C motif chemokine receptor 4
DAPT	tert-Butyl (S)-((2S)-2-[2-(3,5-difluorophenyl)acetamido]propanamido)phenylacetate
DDR	DNA damage response

DIP	2,2'-dipyridyl
DLK1	Delta-like noncanonical Notch ligand 1
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase catalytic subunit
DSB	double-strand breaks
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EPAS1	Endothelial PAS domain protein 1
EPO	Erythropoietin
ERK	Extracellular signal-regulated kinase
FIH	Factor inhibiting HIF
GAPDH	Glyceraldehyde-3-P-dehydrogenase
G-CIMP	Glioma CpG island methylator phenotype
GFAP	Glial fibrillary acidic protein
GLUT1	Glucose transporter 1
GSC	Glioma stem-like cell
HA	Hyaluronan
HAF	Hypoxia-associated factor
HIF	Hypoxia-inducible factor
HRE	Hypoxia response element
Hsp70	Heat shock 70 kDa protein 1A
ICD	Intracellular domain
IDH1/2	Isocitrate dehydrogenase 1/2
IFN	Interferon
IL	Interleukin
JAK	Janus kinase
KLF4	Krueppel-like factor 4
MAPK	Mitogen-activated protein kinase
MGMT	O^6-methylguanine-DNA methyltrasferase
MMP	Membrane-associated metalloprotease
MT1-MMP	Membrane type-1 MMP
mTOR	Mammalian target of rapamycin
N_2	Nitrogen
NAD	N-terminal activation domain
NF1	Neurofibromatosis type 1

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NGFR	Nerve growth factor receptor
NLS	Nuclear localization signal
NODDD	N-terminal oxygen dependent degradation domain
O_2	Oxygen
ODD	Oxygen-dependent degradation
Olig2	Oligodendrocyte transcription factor 2
$p14^{ARF}$	Tumor suppressor ARF
$p16^{INK4\alpha}$	Cyclin-dependent kinase inhibitor 2A
p21	Cyclin-dependent kinase inhibitor 1
p27	Cyclin-dependent kinase inhibitor 1B
p300	300-kilodalton co-activator protein
$p75^{NTR}$	p75 neurotrophin receptor
PAS	Per-Arnt-Sim
PDGF	Platelet-derived growth factor
PDGFRA	Platelet-derived growth factor receptor α
PDLIM1	PDZ and LIM domain protein 1
PHD	Prolyl hydroxylase
PI3K	Phosphatidylinositol 3-kinase
PIK3CA	Phosphatidylinositol 3-kinase subunit alpha
pRB	Retinoblastoma protein
PTEN	Phosphatase and tensin homolog
pVHL	von Hippel-Lindau disease tumor suppressor
Ref-1	DNA-(apurinic or apyrimidinic site) endonuclease
ROS	Reactive oxygen species
Siah2	Seven in absentia homolog 2
Src	Proto-oncogene tyrosine-protein kinase Src
STAT (3)	Signal transducer and activator of transcription (3)
SUMO	Small ubiquitin-like modifier
TAM	Tumor-associated microglia and macrophage
TAPI-2	TNF protease inhibitor 2
TCGA	The Cancer Genome Atlas
TGF	Transforming growth factor
TGM2	Transglutaminase 2

TIF-2	Nuclear receptor coactivator 2
TMI-1	r 4-[[4-(2-butynyloxy)phenyl]sulfonyl]-N-hydroxy-2,2-dimethyl-(3S)-thiomorpholinecarboxamide
TNF	Tumor necrosis factor
TP53	Tumor protein 53
TRE	12-*O*-tetradecanoylphorbol 13-acetate-responsive element
Trk	Tropomyosin-related kinase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Ub	Ubiquitin
VEGF	Vascular endothelial growth factor
VIM	Vimentin
WHO	World Health Organization

Abstract

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All in all, this thesis elucidated aspects of the glioblastoma microenvironment, namely irradiated and hypoxic astrocytes, and the CD44 and $p75^{NTR}$ signaling, that can lead to the development of new targeted therapeutic strategies.

Popular science summary

Glioblastoma is a type of cancer that develops in the brain and mostly affects adults. It is one of the deadliest forms of cancer, and even though patients are treated with surgery, radiotherapy, chemotherapy, and tumor-treating fields, their prognosis is very poor. New therapeutic approaches are therefore needed for the successful treatment of this disease.

It is well-established that glioblastoma is comprised by a combination of cancer cells and cancer-associated 'normal' brain cells. The 'normal' brain cells are found close to the tumor and can be affected by the cancer cells or treatments. The interactions between these cell types, and the environment where the cancer is growing allows the cancer cells to grow, resist therapies, and expand towards the healthy tissue. Moreover, glioblastoma cells themselves are not a uniform population but instead they can be divided into groups with different properties, based on their genetic markup and their location in the tumor. One of these groups, the glioblastoma stem-like cells, exhibits unique properties, such as resistance to treatment and increased tumor formation ability. Interestingly, these cells are found both close to the blood vessels, so in an environment with abundant oxygen, and close to the core of the tumor, where there is very little oxygen. In both cases, the cells activate the mechanism of cellular response to low oxygen and express the hypoxia-inducible factors (HIF-1α and HIF-2α), two proteins that orchestrate this response.

With this thesis we aimed to study the interactions between glioblastoma cells and normal cells under specific conditions of the tumor environment. This provided us with insights on how the population of glioma stem-like cells is changing in these conditions. For this work, we used mouse models and cell-based models of glioblastoma.

In paper I, we found that during treatment of glioblastoma with radiotherapy, the astrocytes, normal cells that are found around and within the tumor, become activated. The activated astrocytes produce a specific protein, TGM2, that when it is incorporated into the protein network of the extracellular space, it induces stem-like cell features in glioblastoma cells. We propose that TGM2 derived from irradiated astrocytes is implicated in the reappearance of the tumor after treatment, because it permanently alters the environment of the cancer. In paper II, we found that astrocytes that are exposed to the low oxygen conditions that are frequently

found in glioblastoma, also become activated. In response to the low oxygen, astrocytes produce a number of proteins that have been associated with tumor growth in previous studies, and they produce a network of proteins that increases the growth and stem-like cell features of glioblastoma cells. These two studies showed that astrocytes, a normal cell of the brain, is affected by irradiation and low oxygen, two conditions that are characterizing almost all glioblastomas. We propose that therapeutically targeting the activation of the astrocytes could lead to improved patient outcomes.

In paper III, we studied the regulation of the glioblastoma stem-like cells by CD44, a molecule that is primarily expressed by these cells and not the rest of the cancer cells. We showed that CD44 interacts with the factor HIF-2α and not HIF-1α, and this interaction leads to an increase in the proportion of glioblastoma stem-like cells, compared to non-stem cells. Finally, we showed that blocking CD44 signaling can decrease the proportion of cells with stem-like properties. In paper IV, we studied the role of another signaling protein, $p75^{NTR}$, in low oxygen conditions in glioblastoma. We found that $p75^{NTR}$ regulates the expression of both HIF-1α and HIF-2α, and it is involved in regulating glioblastoma stem-like cells. Blocking of $p75^{NTR}$ signaling in glioblastoma cells led to a reduction in stem-like cell properties and migration in low oxygen conditions.

In conclusion, our studies emphasize that normal brain astrocytes are affected by treatments, such as radiation therapy, or conditions of the microenvironment, such as low oxygen, in ways that affect the properties of glioblastoma cells. Moreover, we show that blocking signaling pathways activated in the low oxygen conditions of glioblastoma can decrease the proportion of cells with stem-like properties. Overall, our studies further highlight that targeting not only the cancer cells themselves, but also aspects of the environment of the tumor, such as cancer-associated normal cells or low oxygen conditions, is vital for the development of successful therapeutic strategies for glioblastoma patients.

Populärvetenskaplig sammanfattning

Glioblastom är en typ av hjärntumör som oftast drabbar vuxna. Det är en av de allvarligaste typerna av cancer och även om patienter behandlas intensivt med kirurgi, strålning, cellgifter, och tumor-treating fields, är den genomsnittliga överlevnaden endast ca 15 månader efter diagnos. Det finns därmed ett akut behov för nya behandlingsstrategier för att bota patienter med glioblastom.

Glioblastom är en samling av cancerceller och ”normala/friska” celler som finns runt och inom tumören. Dessa ”normala” celler påverkas av cancercellerna och även av behandlingarna. Förhållandet mellan alla dessa celltyper och miljön där tumören finns hjälper cancercellerna att växa, bli resistent mot behandlingar, och även expandera till den normala hjärnan. Dessutom är cancercellerna en heterogen cellgrupp som kan delas upp i olika grupper med olika egenskaper, baserat på genetik och cellernas placering inom tumören. En av dessa grupper som kallas för glioblastom stamceller, har unika egenskaper, som till exempel extrem resistens mot strålning och cellgifter. Glioblastom stamcellerna är även mer aggressiva än andra celler och kan ge upphov till tumörer efter den primära tumören har behandlats bort. Det är uppmärksamt att dessa celler finns både bredvid blodkärl, där finns tillräcklig med syre, och även nära tumörens kärna, där finns det nästan inget syre. I båda fall aktiveras en respons till låg syrehalt hos glioblastom stamcellerna och de producerar två proteiner som organiserar denna respons, hypoxi inducerade faktorer (HIF) 1α och 2α.

Syftet med denna avhandling var att studera förhållandet mellan glioblastom stamcellerna och vanliga hjärncellerna under specifika miljöförhållanden. Detta kommer hjälpa oss att bättre förstå hur glioblastom stamcellerna ändras under dessa miljöförhållanden. Jag har använt mig av musmodeller av glioblastom, samt cellodlingar av normala celler och cancerceller. Avhandlingen innehåller fyra delarbeten.

I Delarbete I visade vi att astrocyter (stjärnceller), normala celler som finns runt och inom glioblastom, påverkas av strålningen och aktiveras. Aktiverade astrocyter producerar TGM2, ett protein som ökar antalet glioblastom stamceller. Vi föreslår att TGM2 som kommer från bestrålade astrocyter ledder till att tumörer kommer tillbaka efter behandlingar. I Delarbete II visade vi att astrocyter aktiveras när de finns i låg syrehalt, en vanlig företeelse i glioblastom. Vid låg syrehalt visar vi att astrocyter producerar flera proteiner som har tidigare förknippas med glioblastom

tillväxt. Våra resultat visade att aktiverade astrocyter producerar ett nätverk av proteiner som hjälper glioblastom celler att expandera och även att utvecklas till glioblastom stamceller. Båda studierna visar att astrocyter påverkas av bestrålning och låg syrehalt, två förhållande som ofta uppstår i glioblastom. Vi föreslår att vi kan hjälpa patienter med glioblastom genom att rikta behandling aktiverade astrocyter.

I Delarbete III studerade vi hur CD44, ett protein som glioblastom stamcellerna producerar, hjälper dem att överleva och växa. Vi visade at CD44 interagerar med HIF2a men inte HIF1a, och interaktionen leder till ett större mängde glioblastom stamceller. Vi visade även att när man blockerar signalen från CD44 så minskade mängde glioblastom stamceller. I Delarbete IV studerade vi vilken roll proteinet $p75^{NTR}$ har vid låg syrehalt i glioblastom. Vi visade att $p75^{NTR}$ reglerar interaktionen mellan HIF1a och HIF2a, och att $p75^{NTR}$ är förknippat med ökat antal glioblastom stamceller. När vi blockerade signalen från $p75^{NTR}$ vid låg syrehalt såg vi en reduktion i mängden glioblastom stamceller och migration av glioblastom celler.

Sammanfattningsvis visar vi att astrocyter, normala celler i hjärnan, påverkas av behandlingar, som till exempel strålning, samt av specifika miljöförhållanden, som till exempel låg syrehalt (Delarbete I och II). Aktiverade astrocyter leder till ökade mängden glioblastom stamceller. Dessutom visar vi att blockering av specifika signaler, som till exempel genom CD44 eller $p75^{NTR}$, vid låg syrehalt, kan minska mängden glioblastom stamceller (Delarbete III och IV). Vår studie visar att vi inte bara behöver behandla glioblastom celler och stamcellerna utan även ”normala” celler i hjärnan som påverkas av tumören eller behandlingarna. Genom att utveckla nya behandlingar kommer vi förlänga och förbättra patienternas liv.

Περίληψη διδακτορικής διατριβής για το ευρύ κοινό

Το γλοιοβλάστωμα είναι ο πιο επιθετικός τύπος καρκίνου του εγκεφάλου. Παρόλο που οι ασθενείς χειρουργούνται, με στόχο την αφαίρεση του όγκου, και λαμβάνουν ακτινοβολίες και χημειοθεραπείες, ο μέσος χρόνος επιβίωσης δεν ξεπερνά τους 8 μήνες. Είναι, συνεπώς, αναγκαία η αναζήτηση νέων θεραπειών για την αντιμετώπιση αυτής της ασθένειας.

Ενώ αρχικά, το γλοιοβλάστωμα, όπως και άλλοι καρκίνοι, θεωρούνταν μία ενιαία μάζα από παρόμοια κύτταρα, σήμερα γνωρίζουμε πως αποτελείται από ένα συνδιασμό καρκινικών και μη καρκινικών κυττάρων. Οι σχέσεις μεταξύ αυτών των κυττάρων και του περιβάλλοντος στο οποίο βρίσκεται ο όγκος, επιτρέπουν στα καρκινικά κύτταρα να πολλαπλασιάζονται, να αντιστέκονται σε θεραπείες, και να διεισδύουν στον φυσιολογικό εγκεφαλικό ιστό. Επιπλέον, τα ίδια τα καρκινικά κύτταρα αποτελούν έναν ετερογενή πληθυσμό κυττάρων, με διαφορετικούς υποτύπους, ο καθένας με μοναδικές ιδιότητες. Αυτοί οι υπότυποι καρκινικών κυττάρων καθορίζονται αφενός από το γενετικό υπόβαθρο των κυττάρων και αφετέρου από τα μηνύματα που δέχονται από το περιβάλλον στο οποίο βρίσκονται. Ένας από αυτούς τους υποτύπους είναι τα καρκινικά βλαστοκύτταρα, τα οποία χαρακτηρίζονται από αυξημένη αντοχή σε θεραπείες (ακτινοβολία και χημειοθεραπεία) και είναι υπεύθυνα για την επανεμφάνιση του όγκου μετά τη θεραπεία. Τα κύταρα αυτά εντοπίζονται είτε κοντά στα αιμοφόρα αγγεία, δηλαδή σε περιοχές του όγκου με υψηλή περιεκτικότητα σε οξυγόνο, είτε κοντά σε περιοχές υποξίας, δηλαδή περιοχές του όγκου με σχεδόν μηδενική περιεκτικότητα σε οξυγόνο. Και στις δύο αυτές περιπτώσεις, τα καρκινικά βλαστοκύτταρα ενεργοποιούν τον κυτταρικό μηχανισμό αντιμετώπισης της υποξίας και εκφράζουν τις πρωτεΐνες που ρυθμίζουν αυτό το μηχανισμό, τους λεγόμενους επαγώγιμους με υποξία παράγοντες (HIF-1α και HIF-2α).

Σε αυτή την διατριβή μελετήσαμε τις σχέσεις μεταξύ καρκινικών και φυσιολογικών κυττάρων του εγκεφάλου κάτω από δεδομένες συνθήκες, συγκεκριμένα υπό το πλαίσιο της ακτινοβολίας και της υποξίας. Σκοπός μας ήταν να κατανοήσουμε τον τρόπο συντήρησης των καρκινικών βλαστοκυττάρων υπό αυτές τις συνθήκες. Για τον σκοπό αυτό, χρησιμοποιήσαμε ζωικά μοντέλα γλοιοβλαστώματος και κυτταρικές σειρές καρκινικών και φυσιολογικών κυττάρων.

Στο 1ο άρθρο, ανακαλύψαμε πως κατά την ακτινοβόληση του γλοιοβλαστώματος, τα αστροκύτταρα, φυσιολογικά εγκεφαλικά κύτταρα που βρίσκονται γύρω από και μέσα στον καρκινικό όγκο, ενεργοποιούνται. Τα ενεργοποιημένα αστροκύτταρα παράγουν την πρωτεΐνη TGM2, που όταν ενσωματώνεται στο πρωτεϊνικό δίκτυο που βρίσκεται μεταξύ των κυττάρων, προκαλεί αύξηση στον αριθμό των καρκινικών βλαστοκυττάρων. Επίσης, ανακαλύψαμε πως η TGM2 που παράγεται από τα ακτινοβολημένα και ενεργοποιημένα αστροκύτταρα συμμετέχει στην επανεμφάνιση του καρκίνου μετά την θεραπεία, καθώς προξενεί μόνιμες αλλαγές στο περιβάλλον του καρκίνου που ευνοούν την μελλοντική επανεμφάνισή του. Στο 2ο άρθρο, ανακαλύψαμε πως τα αστροκύτταρα ενεργοποιούνται όταν βρίσκονται σε συνθήκες υποξίας, μία συνθήκη που πολύ συχνά χαρακτηρίζει το γλοιοβλάστωμα. Παρουσία υποξίας, τα αστροκύτταρα παράγουν πρωτεΐνες που στο παρελθόν έχουν συσχετισθεί με την ανάπτυξη του γλοιοβλαστώματος. Επιπλέον τα υποξικά αστροκύττρα παράγουν ένα δίκτυο πρωτεϊνών που, όπως και στην περίπτωση των ακτινοβολημένων αστροκυττάρων, υποστηρίζει την ανάπτυξη των καρκινικών βλαστοκυττάρων. Αυτά τα δύο άρθρα μας δείχνουν πως φυσιολογικά εγκεφαλικά κύτταρα, τα αστροκύτταρα, ενεργοποιούνται από την ακτινοβολία και την υποξία, δύο συνθήκες που συχνά χαρακτηρίζουν το γλοιοβλάστωμα, και συντηρούν τα επίπεδα καρκινικών βλαστοκυττάρων στο γλοιοβλάστωμα. Στο 3ο άρθρο, μελετήσαμε τη ρύθμιση των καρκινικών βλαστοκυττάρων από την πρωτεΐνη CD44, που κυρίως εκφράζεται στα καρκινικά βλαστοκύτταρα και όχι σε άλλα υποείδη καρκινικών κυττάρων. Ανακαλύψαμε πως το CD44 αντιδρά συγκεκριμένα με τον HIF-2α και όχι με τον HIF-1α, τόσο σε συνθήκες υποξίας όσο και σε περιοχές δίπλα στα αιμοφόρα αγγεία. Αυτή η αντίδραση οδηγεί στην συντήρηση και αύξηση των καρκινικών βλαστοκυττάρων. Επίσης, είδαμε πως η μείωση της δράσης του CD44 οδήγησε σε μειωμένα επίπεδα καρκινικών βλαστοκυττάρων. Στο 4ο άρθρο, μελετήσαμε μία ακόμα πρωτεΐνη, την $p75^{NTR}$, σε συνθήκες υποξίας στο γλοιοβλάστωμα. Βρήκαμε πως η $p75^{NTR}$ ρυθμίζει την έκφραση των HIF-1α και HIF-2α εξίσου, και πως επίσης συμμετέχει στην συντήρηση των καρκινικών βλαστοκυττάρων. Μείωση της δράσης του $p75^{NTR}$, υπό συνθήκες υποξίας, οδήγησε σε μειωμένα επίπεδα καρκινικών βλαστοκυττάρων.

Συμπερασματικά, οι μελέτες μας έδειξαν ότι τα φυσιολογικά αστροκύτταρα επηρεάζονται από θεραπείες, όπως η ακτινοβολία, και από συνθήκες του καρκινικού περιβάλλοντος, όπως η υποξία. Τα ενεργοποιημένα αστροκύτταρα επηρεάζουν τα ποσοστά καρκινικών βλαστοκυττάρων στο γλοιοβλάστωμα. Επίσης, οι μελέτες μας έδειξαν πως η μείωση της δράσης συγκεκριμένων πρωτεϊνών που ενεργοποιούνται σε συνθήκες υποξίας, μπορεί να μειώσει τα ποσοστά καρκινικών βλαστοκυττάρων στο γλοιοβλάστωμα. Συνολικά, οι μελέτες μας αποδεικνύουν πως για την αποτελεσματική αντιμετώπιση του γλοιοβλαστώματος, θα πρέπει να στοχεύσουμε στην καταπολέμιση όχι μόνο των καρκινικών κυττάρων, αλλά και των φυσιολογικών κυττάρων που σχετίζονται με αυτά.

Cancer

Cancer, a disease that affects more than 19 million people annually, is one of the leading causes of death worldwide [1]. It has therefore attracted the interest of scientists throughout the centuries. From the Greek physician Hippocrates (460-370 BC) who described crab-like malignant growths and coined the terms carcinoma and cancer [2, 3] to the gradual establishment of clinical oncology [4], scientists have long been trying to characterize, understand, and target this disease. This thesis builds on the work of others and further contributes to our understanding of the biology of one type of cancer, namely of glioblastoma.

Cancer development

The transition from a normal cell to a malignant state requires the acquisition of several traits. These traits have been elegantly summarized by Hanahan and Weinberg and include among others uncontrolled growth, cell death evasion, changes in cell metabolism, and immune cell evasion [5, 6]. In the apex of the hallmarks of cancer, as they termed these malignant traits, lies the increased genomic instability and the tumor-promoting inflammation [5, 6], indicating that tumors are maintained not only by intrinsic characteristics of cancer cells but also by changes inflicted by stromal cells. Understanding the cross-talk between cancer cells and stromal cells might be one way forward in the discovery of new cancer treatments.

Glioblastoma

Epidemiology, treatment, and prognosis

Gliomas are brain tumors of glial origin that represent more than 80% of all malignant primary brain tumors [7]. Gliomas include non-diffuse and diffuse subtypes, with the latter being more frequent [8]. Diffuse gliomas include astrocytomas, oligodendrogliomas and mixed tumors (oligoastrocytomas) [8]. Glioblastoma is the most aggressive and common primary diffuse astrocytic glioma [8], with a dismal prognosis. The median survival is only 8 months after diagnosis and the 5-year survival is 7.2% [7]. Glioblastoma affects primarily older adults and is more prevalent among Caucasian males [7].

Despite our increasing understanding of glioblastoma biology, only few additions in the standard of care have been made over recent years. Patients diagnosed with glioblastoma undergo surgery, with the aim of maximal safe resection, followed by temozolomide administration with concomitant radiation therapy (a total of 60 Gy over the course of 30 days), followed by temozolomide alone [9]. Because of the infiltrative nature of glioblastoma, complete surgical resection is difficult. Patients who undergo surgery and have complete tumor resection survive longer than those who do not [9, 10]. The addition of radiation and finally temozolomide in the treatment regimen further extended patient survival [9, 11]. The addition of tumor-treating fields to maintenance temozolomide treatment, a novel noninvasive therapy that uses alternating electrical fields to disrupt cancer cell division, has further improved the survival of glioblastoma patients [12]. Despite these treatment modalities, tumors invariably recur, within 7 months after initiation of the treatment, typically within 2 cm of the original tumor volume and within the original field of radiation [13]. There is no standard-of-care regimen after recurrence and the recurrent tumor is more often than not insensitive to further treatments, including radiation and temozolomide repeat treatments [14]. Enrollment in clinical trials is frequently the main option for patients with recurrent glioblastoma, although recently, surgery has been suggested to improve survival in patients who undergo a second complete resection [15].

Favorable clinical prognostic factors for glioblastoma patients include young age, high extent of surgical resection, and a good Karnofsky performance status, a measure of patients' functional ability [16]. Favorable molecular prognostic factors include O^6-methylguanine-DNA methyltrasferase (*MGMT*) promoter methylation,

isocitrate dehydrogenase 1 (*IDH1*) mutations, and the presence of a glioma CpG island methylator phenotype (G-CIMP) [17-19]. *IDH1* mutations occur in less than 4% of primary glioblastomas and are associated with slower tumor growth [20]. Patients with mutant *IDH1* also tend to be younger, and have a G-CIMP phenotype [19, 21, 22]. Methylation of the *MGMT* promoter occurs in approximately 40% of glioblastoma patients and leads to silencing of the *MGMT* gene and subsequently reduced DNA repair and increased susceptibility to alkylating agents. As a result, patients with methylated *MGMT* respond better to chemotherapy treatments, such as temozolomide [17, 23, 24].

Histopathological and molecular features of glioblastoma

The most defining histopathological feature of glioblastoma is its infiltrative nature. Tumor cells invade into the neighboring tissue and even cross the corpus callosum, seeding the opposite hemisphere and giving rise to multifocal lesions. Glioblastoma lesions have an astroglial appearance, and are characterized by high mitotic activity, microvascular proliferation and pseudopalisading necrosis [25].

Historically, glioblastoma was diagnosed as a World Health Organization (WHO) grade IV astrocytoma; however, in 2016 WHO proposed a new classification for diffuse gliomas based on the presence or absence of mutations in *IDH1* and *IDH2* [8]. Based on *IDH* mutation status, glioblastomas are now classified as *IDH*-wildtype and *IDH*-mutant [8]. *IDH*-mutant glioblastoma corresponds to what was previously diagnosed as secondary glioblastoma, meaning glioblastoma that progresses from a lower grade lesion, while *IDH*-wildtype glioblastoma corresponds to what was diagnosed as grade IV astrocytoma, or glioblastoma multiforme [8]. As mentioned above, *IDH* mutation status affects patient survival and patients that harbor *IDH* mutations have a better prognosis compared to those with *IDH*-wildtype [26].

Several genetic aberrations have been identified in glioblastoma (Table 1). Epidermal growth factor receptor (EGFR) signaling is frequently altered in glioblastoma (Figure 1) and amplification of the receptor occurs in approximately 40% of primary glioblastomas [27]. Another frequent *EGFR* aberration is the *EGFRvIII* mutation that leads to constitutive receptor activation [27, 28]. Amplification of *EGFR* or constitutive activation of the receptor has been linked to decreased patient survival [29]. Platelet-derived growth factor receptor α (PDGFRA) is another receptor tyrosine kinase that is amplified in glioblastoma [27, 28]. Activated EGFR or PDGFRA leads to activation of the PI3K/Akt/mTOR, the Ras/MAPK/ERK, and the JAK/STAT pathway, all of which converge to increased cell proliferation and cell survival [30]. The phosphatase and tensin homolog

(*PTEN*) gene is also frequently mutated or lost as part of a frequent chromosome 10q loss in glioblastoma [27]. PTEN inhibits Akt activation and as a result, mutations in *PTEN* result in increased cell proliferation [27]. Neurofibromatosis type 1 (*NF1*) is another gene acting as a tumor suppressor in glioblastoma. NF1 acts as a negative regulator of Ras and therefore regulates the activation of the Akt/mTOR pathway [27]. *TP53* mutations are frequent in glioblastomas, particularly in secondary glioblastomas [31]. Loss of $p14^{ARF}$, a regulator of p53 activity, is also frequent in glioblastoma [32] and leads to loss of p53 activity. Loss of p53 function is associated with increased glioma cell invasion, migration, and proliferation [33]. Finally, $p16^{INK4\alpha}$ deletions are relatively frequent in glioblastoma [34] and lead to deregulation of the cell cycle [35].

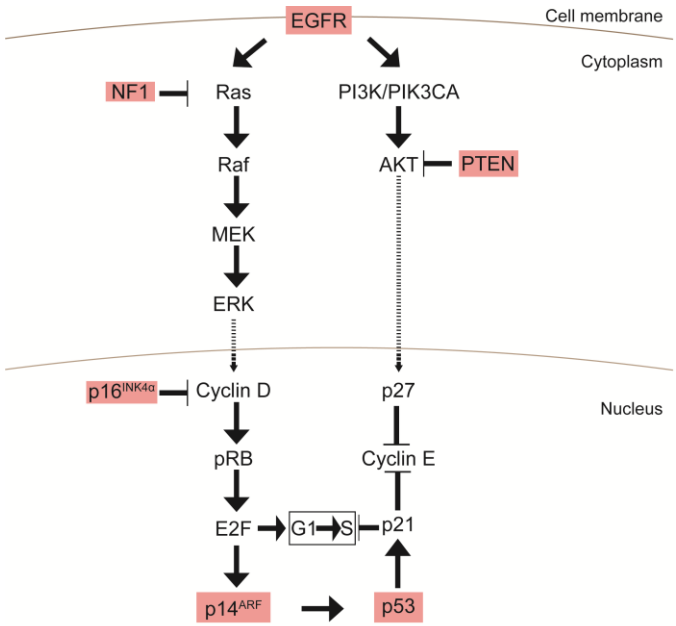

Figure 1 Common mutations found in glioblastoma along the EGFR signalling pathway
The EGFR pathway is frequently altered in glioblastoma, with several genetic abberations found along its axis. The most frequent mutations are marked in red. ERK: extracellular signal-regulated kinase, pRB: retinoblastoma protein, p53: tumor protein 53, Akt: protein kinase B, PI3K: phosphatidylinositol 3-kinase, PIK3CA: PI3K subunit alpha

In 2010, Verhaak et al. stratified glioblastoma patients from The Cancer Genome Atlas (TCGA) into four molecularly distinct subtypes; proneural, classical, mesenchymal, and neural [22]. The authors reported that generic aberrations or altered gene expression in *PDGFRA/IDH1*, *EGFR*, and *NF1* characterize the proneural, classical, and mesenchymal subtypes, respectively [22], while the neural signature was later found to be contamination from normal cells [36]. Despite the

authors proposing that these molecular subtypes could provide new avenues for personalized treatments, no difference seems to exist in the clinical response or survival of patients belonging to different glioblastoma subtypes. Only the proneural subtype was associated with a survival advantage, but that was due to the prevalence of the G-CIMP phenotype in this group [21]. The reason for this lack of prognostic value of the molecular subtypes could be the high degree of intratumoral heterogeneity that characterizes glioblastomas and the fact that lesions from single patients harbor mutations associated to all of the above-mentioned subtypes [36, 37]. As a result, no subtype-specific treatment can eliminate all tumor cells. Several elegant studies have showed that a high degree of heterogeneity characterizes glioblastoma lesions [36-40]. These studies showed that cells from all subtypes can be found in individual patients' lesions [37] and importantly they provide evidence that cells from one subtype can convert to another subtype [36, 38]. Moreover, recurrent tumors frequently exhibit a different transcriptional profile compared to the primary lesions [36, 41-43]. These new insights in the phenotypic heterogeneity of glioblastoma explain the difficulty in identifying subtype-specific therapeutic targets and underlines the need for new approaches in the search for therapies for this disease.

Table 1 Frequently occuring mutations in glioblastoma
(Adapted from [27] and [28])

Gene	Frequency in primary glioblastoma
10q loss	> 70%
$p16^{INK4a}$ loss	~ 52%
$p14^{ARF}$ loss	~ 49%
EGFR amplification	~ 40%
TP53 mutation	~ 30%
PTEN mutation	~ 30%
NF1 mutation	~ 20%
PDGFRA amplification	~ 16%
IDH1 mutation	~ 5%

Cancer stemness

The clonal evolution and cancer stem cell models

One cannot discuss about cancer and cancer development without referring to the two models of cancer development, namely the clonal evolution model and the cancer stem cell model.

For years, it was accepted that tumors form when cells acquire new properties due to the occurrence of successive mutations (genetic hits). Responsible for this accumulation of mutations is the genetic instability that characterizes cancer cells. The product of this process is the generation of a heterogenous lesion comprised by various clones, where each clone has acquired a distinct set of mutations [44]. For instance, various genetic aberrations characterize glioblastoma and several of these aberrations can be found in one lesion, giving rise to intratumoral heterogeneity.

This clonal evolution model of cancer development and progression has been challenged by the cancer stem cell model. The cancer stem cell model proposes that cancer cells with stem-like properties are found in the apex of the cell hierarchy in a tumor. First identified in hematopoietic malignances [45], cancer stem-like cells (CSCs) are a self-renewing, quiescent, and therapy resistant cell population that can give rise to several cancer cell types and thus contributes to tumor heterogeneity [46, 47]. CSCs have since been described in breast, pancreas, and colon cancers [48-50], and, importantly and relevant to this thesis, in glioma [51].

In pancreatic cancer and in glioma, studies have shown the importance of the identified CSC population for both migration and tumor growth or tumor initiation [52, 53] .These studies suggest that not all tumor cells have the same potential to metastasize, evade treatment or reconstitute a tumor in animal models, suggesting that intratumoral heterogeneity arises from the existence of a CSC pool that differentiates into cells with different phenotypes.

Support for the cancer stem cell model comes not only from identification and isolation of the CSC population from various cancers, but also from studies that compare the genetic profile of primary and recurrent tumors. In glioma, post-treatment, recurrent tumors often harbor different clonal profiles compared to the ones observed at diagnosis [41-43]. According to the clonal evolution model, recurrent and post-treatment tumors arise after part of the primary tumor survives treatment and continues to grow. Although these residual cells accumulate

additional mutations, many of the mutations identified in the surviving clones of the primary tumor are maintained in the recurrence. As a result, there is a high degree of similarity between primary and recurrent tumors. According to the cancer stem cell model, recurrent tumors arise from dormant cancer cells, the CSCs. In this process, CSCs accumulate mutations and give rise to recurrent tumors that only share a few common mutations with the primary lesion, the ones originally found in the CSCs. Evidence for the existence of both models in glioblastoma patients has been found by sequencing matched primary and recurrent samples [42].

The clonal evolution and cancer stem cell models are just two efforts into understanding the phenomena surrounding tumor growth dynamics, but neither model can fully explain the observed complexity of tumor phenotypes, arising both before and after treatment. A more unifying theory is combining the two models in a way that explains both the presence of several clones within one tumor but also the presence of cells with stem-like properties [46]. According to this theory, tumors are comprised by several clones, each one contributing to the intratumoral genetic heterogeneity. The cells within each clone, however, are functionally different, with some being terminally differentiated (bulk tumor cells) and others remaining dormant (CSCs) (Figure 2). Interestingly, one study of glioma found that differentiated tumor cells communicate with stem-like cells and this communication contributed to the maintenance of the stem-like cell pool, a process that led to increased tumor growth [54]. This unifying theory suggests that genetic diversity found in clones, epigenetic regulation, and factors found within the tumor microenvironment all affect the stemness properties of cancer cells and thus contribute to tumor heterogeneity.

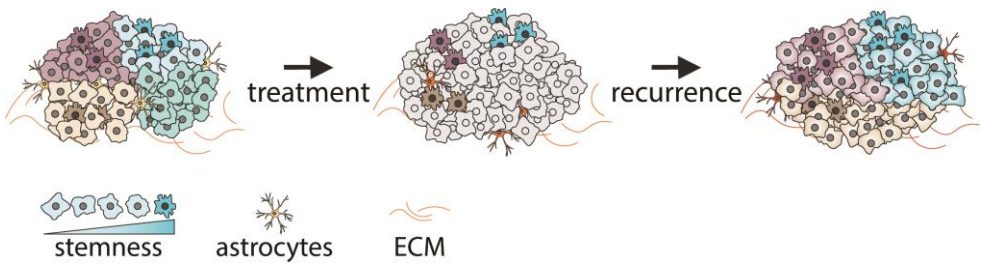

Figure 2 Unification of the clonal evolution and cancer stem cell models
Each clone that is generated as a result of genetic diversity is comprised of bulk and stem-like cancer cells, surrounded by stromal cells, such as astrocytes, and extracellular matrix (ECM). Some clones disappear from the recurrent tumor due to their sensitivity to treatments. After treatment, the tumor is repopulated thanks to the presence of resistant cells within the original mass. New mutations can arise during treatment that further increase the genetic diversity of the tumor.

The concept of phenotypic plasticity has added another layer of complexity in the studies of cancer stemness. Phenotypic plasticity directed either by intrinsic mechanisms or initiated due to microenvironmental changes leads to shifts in cellular states, with direct effects on disease progression and patient survival. In

glioblastoma, single cell analyses showed that intratumoral heterogeneity arises from the presence of four cellular states which exhibit high plasticity [38]. Dirkse et al. reported that isolated glioma cells reconstituted the original heterogeneity *in vitro* and that the phenotypic heterogeneity of glioma cells is affected by factors such as hypoxia or the *in vivo* tumor microenvironment [39]. Similarly, Neftel et al. showed that when glioma cells of a single state are implanted in mice, they give rise to tumors that recapitulate the complexity of cellular states found in the primary tumor [38]. The authors also reported a dependency of individual cellular states on microenvironmental factors [38].

It is therefore becoming increasingly clear that tumors are not static systems comprised of groups of cells that exhibit similar genetics. On the contrary, tumors are complex ecosystems where cancer cells, CSCs and stromal cells all contribute to disease progression and therapy resistance and eventually influence patient outcomes.

Cancer stem-like cell niches

Similar to normal stem cells, cancer stem cells are thought to be enriched in distinct microenvironments of the tumor. These areas are termed CSC niches and consist of bulk tumor cells, CSCs, and tumor-associated stromal cells. Cancer-associated fibroblasts, mesenchymal stem cells, and immune cells are amongst the stromal cells found in CSC niches and interact with CSC themselves [55].

In glioblastoma there are three well-characterized stem cell niches; the invasive, the perivascular, and the perinecrotic niche [56]. Because high grade gliomas are characterized by increased vasculature and extensive hypoxia, those niches are well-studied in the context of stemness maintenance and will be discussed in a later chapter.

Cancer stem-like cell properties

CSCs are historically defined by properties that also characterize their normal tissue counterparts; quiescence, scarcity, and self-renewal [57].

The definition of a CSC has evolved since the first studies on stemness. Quiescence was until recently thought to be an integral trait of CSCs. Since cell division can introduce errors in the DNA, stemness has been associated with relatively slow-cycling cells. However, recent studies of cellular states in gliomas show that stem-like cells harbor gene signatures of cycling cells and might be more proliferative than initially thought [38, 58-60]. The scarcity of the CSC population has also been

challenged. A typical example is melanoma, where 27% of unselected human melanoma cells formed tumors *in vivo*, in contrast to many other tumor types, where less than 1% of the cancer cells have this potential [61]. This suggests that melanomas have a shallow heterogeneity and many of the tumor cells are in fact CSCs. Moreover, as the authors comment, the technical aspects of the *in vivo* tumorigenicity assay can greatly influence the results, so studies calculating the frequency of CSCs should be interpreted with caution [61].

Several methods have been used to evaluate the stemness phenotype of cancer populations. The definitive stemness assay is the *in vivo* tumorigenicity assay. Serial transplantation experiments in mice can identify cell populations that are able to self-renew and give rise to tumors that recapitulate the disease. Therefore, the assay presents the gold standard for verifying stem cell states. However, these assays are both time consuming and costly since they involve the maintenance of mice for several generations. Therefore, *in vitro* assays have been established to aid CSC research. Broadly used *in vitro* assays include the sphere formation, the limiting dilution self-renewal, and the radiation and chemotherapy resistance assays, as well as the drug efflux, differentiation capacity, and stem cell marker expression analysis.

Sphere formation assays have been extensively used in the field of neuroscience to study neural stem cell biology. In cancer research, such assays address the ability of cancer cells to self-renew and are used as a proxy for stemness. Size and number of spheres have been used as readouts of sphere assays. However, sphere size can be influenced by growth factor signaling, and also reflects the proliferation potential of the cells. Sphere number is therefore a more reliable measure of stemness. For a more robust assessment of self-renewal and stemness, the secondary or tertiary sphere formation potential can be evaluated. These assays are simple to set up and interpret; however, careful consideration should be given to the experimental design and interpretation of the results. Clonality during cell plating is a crucial parameter of the sphere formation assays as free-floating spheres can easily form aggregates. An important note is that sphere formation assays detect only cells with stem cell properties that are proliferating. As such, these assays cannot detect quiescent stem cells that lack the ability to proliferate *ex vivo* [62]. More recently, the stem cell field has adopted the use of the limiting dilution self-renewal assay, an adaptation of the sphere formation assay. This assay offers the advantage of more robust statistical testing of the stem-cell hypothesis, and has the statistical power to analyze multiple data sets [63].

CSCs are thought to be resistant to radiation and chemotherapy compared to non-stem-like, bulk tumor cells. These characteristics are a result of several properties of CSCs such as slower proliferation relative to bulk tumor cells, increased DNA repair mechanisms, upregulation of drug-efflux pumps, and increased ROS clearance [64-68]. The colony formation after radiation or drug treatment, and the

side population assay, which evaluates the drug efflux capacity of a cell population, are used to study these characteristics of CSCs *in vitro*.

Stemness assays are generally performed in the absence of serum, to avoid cell differentiation. In the context of brain tumors, to maintain and enrich for brain stem-like cells, the cells are maintained without serum, in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) [69]. Withdrawal of the growth factors and addition of serum in the culture medium leads to differentiation. Assessing differentiation of CSCs towards the neuronal, astrocytic, and oligodendrocytic lineages addresses the multipotency of these cells in culture.

Several efforts have been made to identify reliable and specific stem cell markers. Over the years several stem cell populations have been prospectively isolated based on marker expression. CD133, CD44, nestin, Nanog, SOX2, Oct4, signal transducer and activator of transcription 3 (STAT3), and glial fibrillary acidic protein (GFAP) have all been used in glioma stem-like cell (GSC) studies as stemness markers [70]. However, using markers to study cancer stemness does not account for the profound plasticity that characterizes tumor cells, as mentioned above. Experimental evidence from breast and colon cancer shows that non-stem-like cells, as defined by the lack of stem cell marker expression, can convert towards a stem-like state [71, 72]. Similarly, Dirkse et al. showed that the expression of commonly used glioma stemness markers is subject to changes in response to microenvironmental signals [39].

Overall, it has become increasingly clear that CSCs are phenotypically plastic, and no single assay is sensitive enough to reliably identify this cell type. As a result, several approaches and a combination of functional assays and stem cell marker expression should be used when studying cancer stemness.

Tumor hypoxia

In mammalian physiology, oxygen has a crucial role in maintaining life. Reductive and oxidative reactions that are at the basis of oxygen physiology are fundamental in mitochondrial and cytosolic enzymatic functions. Not only oxygen metabolism but also by-products of it, such as reactive oxygen species (ROS), are involved in several processes.

Defining physiological oxygen levels in tissues can be challenging. Healthy tissues vary greatly in what constitutes physiological oxygen levels and even within a given tissue, oxygen levels can vary greatly. For instance, oxygen concentrations from 12.5% to 2.5% have been reported for the healthy brain [73, 74]. In the lab, modelling physiological oxygen tension has also proven challenging. Most *in vitro* experiments are conducted at 37 ºC, 5% CO_2, 74% N_2, and 21% O_2. This oxygen tension has been historically referred to as normoxia and has been used as the normal control in studies of oxygen regulation [75, 76]. However, virtually all tissues have oxygen levels much lower than 21%. Already in the arteries, the oxygen levels drop to approximately 10% [75, 76]. When oxygen diffuses from the blood vessels to the various tissues it reaches approximately 100 to 200 μm away from the end capillaries [77]. In most tissues oxygen levels are at an average of 6.1%, therefore 5% oxygen has been proposed as a more relevant oxygen tension that represents physiological oxygen levels, or physoxia [76].

The lack of adequate oxygen is defined as hypoxia. Hypoxia can be divided between physiological and pathological hypoxia. Physiological hypoxia is the transient decrease in oxygen tension that leads to the activation of the hypoxia-response pathway and the subsequent return to physoxia. Pathological hypoxia describes the chronic reduction in oxygen levels due to the inability of the cells to return to physoxia [76].

Hypoxia-Inducible Factors

As oxygen is imperative to maintaining cell homeostasis, oxygen sensing is also important. In fact, the discovery of the mechanisms regulating oxygen sensing and cellular adaptations to hypoxia was awarded the Nobel Prize in Physiology or Medicine 2019. The work associated with the Nobel Prize revealed that the hypoxic

response is highly regulated by a set of transcription factors called hypoxia inducible factors (HIFs).

Originally, HIF-1α was identified for binding to a hypoxia response element (HRE) in the human erythropoietin (*EPO*) gene in hypoxia [78]. It was subsequently shown that this binding was not specific to *EPO* regulation in kidney and liver cells but that it might be extended to various genes and cell types [79]. Soon after the discovery of HIF-1α, HIF-2α or endothelial PAS domain protein 1 (EPAS1) was identified and was found to bind the same HRE as HIF-1α [80, 81].

HIFα subunits are basic-helix-loop-helix proteins containing a PAS domain [82, 83], that is involved in subunit dimerization, in DNA binding, and in signal transduction [83, 84]. HIFα subunits have two oxygen dependent degradation (ODD) domains, an N-terminal ODD domain (NODDD) and a C-terminal ODD domain (CODDD), and two transactivation domains, the N-terminal activation domain (NAD) and the C-terminal activation domain (CAD) [85]. Finally, the HIFα subunits have two nuclear localization signals (NLS) [86] (Figure 3). HIFα subunits form heterodimers with the constitutively expressed HIFβ (also known as aryl hydrocarbon receptor nuclear translocator, ARNT) [78, 87]. HIF-1α and HIF-2α exert their transcriptional activity under hypoxic conditions by interacting via the CAD with the central transcriptional co-activator CBP/p300, and other co-activators such as Src, TIF-2, and Ref-1 [88-90].

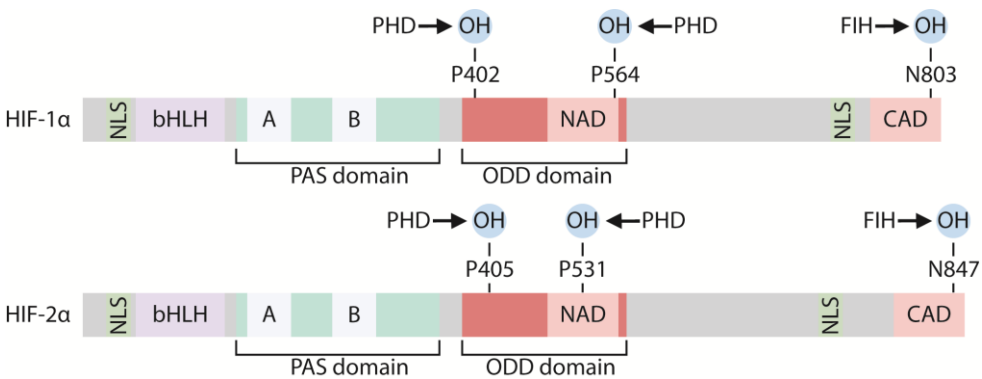

Figure 3 HIFα subunit structure
There is great homology in the structure of HIF-1α and HIF-2α. Both subunits are basic helix-loop-helix (bHLH) proteins with conserved nuclear localization signals (NLS), a PAS domain, an oxygen-dependent degradation (ODD) domain containing the NAD, and CAD. They differ in the sites for hydroxylation by the prolyl hydroxylases (PHD) and the asparaginyl hydroxylase (FIH).

Finally, there is a third HIFα subunit, HIF-3α that is much less studied. HIF-3α is also regulated in an oxygen-dependent manner and has an ODD [91]. A splice variant of HIF-3α, induced by hypoxia, might be acting as a negative regulator of hypoxia-dependent gene expression [92, 93].

Regulation of HIFs

The regulation of HIF activity is dependent on the stabilization and transcriptional activation of the HIFα subunits. Two types of oxygen-dependent hydroxylases are at the basis of the regulatory mechanism. Three prolyl hydroxylase domain (PHD1-3) hydroxylases are responsible for the hydroxylation of conserved prolyl residues in the NODDD and CODDD, and one asparaginyl hydroxylase, factor inhibiting HIF (FIH), hydroxylates an asparaginyl residue in the CAD (Figure 3). These hydroxylases belong to the family of non-haem, Fe^{2+}-dependent, 2-oxoglutarate-dependent oxygenases. During the enzymatic reaction, water is used as an oxygen carrier to couple oxygen to the hydroxylation substrate, in this case HIFα, and to 2-oxoglutarate to give succinate and CO_2. Interestingly, PHD2 and PHD3 are induced by hypoxia, indicating the existence of a feedback loop for the regulation of cellular responses to hypoxia [85].

The hydroxylation of the prolyl and asparaginyl residues leads to differential regulation of HIF activity. Prolyl hydroxylation by the PHDs regulates the proteasomal degradation of HIFα subunits. Hydroxylated HIFα in the conserved prolyl residues is recognized by the highly specific von Hippel-Lindau disease tumor suppressor (pVHL), a part of a multi-component ubiquitin ligase. After ubiquitination, HIFα is targeted for degradation by the ubiquitin-proteasome pathway [94-97]. Interestingly, asparaginyl hydroxylation by FIH does not affect the stability of HIFα but instead leads to the inhibition of its transcriptional activity. Hydroxylated HIFα in the conserved asparaginyl residues can no longer interact with CBP/p300 and is therefore a poor inducer of transcription [98].

In normoxic conditions, the PHDs hydroxylate the HIFα subunits, targeting them for proteasomal degradation. Subunits that escape degradation are hydroxylated by FIH and are transcriptionally dampened. In hypoxic conditions, the PHDs and FIH can no longer hydroxylate HIFα, the subunits escape proteasomal degradation and translocate to the nucleus. There, they interact with HIFβ and their transcriptional co-activators and mediate downstream signaling (Figure 4).

The enzymatic reactions that regulate HIFα stability and transcriptional activity are highly dependent on oxygen. However, the PHDs and FIH have different affinities to oxygen and can therefore exert their regulatory function on HIFα to different degrees in varying oxygen tensions [99]. More specifically, FIH has a higher affinity for oxygen and can inhibit HIFα transcriptional activity even at the low oxygen tension of 1% [100]. Interestingly, HIF-1α and HIF-2α have different affinities for FIH, possibly due to the presence of a specific amino acid difference in the CAD of the two HIFs [101]. These differences in FIH affinity might be translated in differential gene expression, however there is still no consensus on whether some genes are specific HIF-1α or HIF-2α targets.

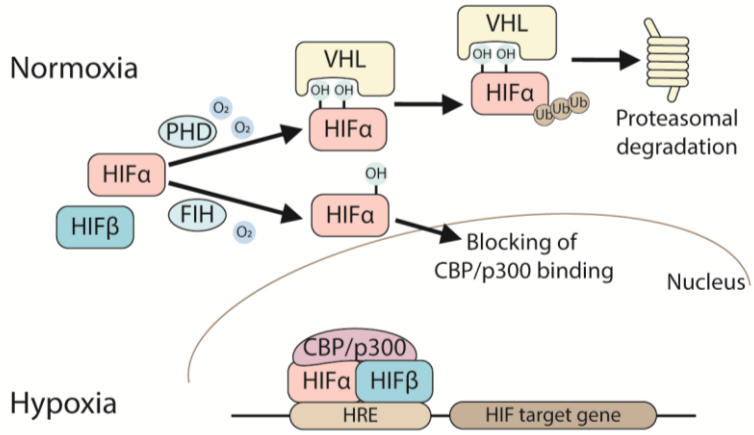

Figure 4 Regulation of HIFα subunits under normoxia and hypoxia
HIFα subunits are regulated in normoxic conditions by the PHDs and FIH. Hydroxylation of the HIFα subunits by the PHDs leads to ubiquitination (Ub) and proteasomal degradation. Hydroxylation by FIH blocks the binding of the transcriptional coactivator CBP/p300 and decreases HIF transcriptional activity. In hypoxia, HIFα is stabilized, translocates to the nucleus where it interacts with the constitutively expressed HIFβ and with CBP/p300, and binds to HREs of target genes, leading to hypoxia-regulated gene transcription.

Although the main mechanism for HIF regulation is hydroxylase-dependent, other regulatory mechanisms have been identified. In low oxygen tensions (between 2% to 5%), it has been shown that PHD1 is degraded by the ubiquitin ligase seven in absentia homolog 2 (Siah2), in a process dependent on Akt activation, leading to HIF stabilization [102]. Hypoxia-associated factor (HAF) is a nuclear protein, acting as an E3 ligase, that leads to HIF-1α but not HIF-2α degradation independently of pVHL or oxygen tension [103]. Hsp70 and E3 ubiquitin-protein ligase CHIP also lead to the ubiquitination and proteasomal degradation preferentially of HIF-1α, under prolonged hypoxia [104]. Small ubiquitin-like modifier (SUMO)ylation is another mechanism that regulates HIFα stability. SUMOylation of HIF-1α and HIF-2α leads to binding of pVHL, independent of the presence of prolyl hydroxylation [105, 106].

In cancer, the HIFs are stabilized not only due to the lack of oxygen that frequently characterizes tumor lesions, but also due to the dysregulation of signaling pathways and mutations in tumor suppressor genes. For instance, it has been reported that loss of *PTEN* in glioblastoma cell lines leads to increased expression of HIF target genes, possibly by Akt-mediated stabilization of the HIFs [107]. In support of this finding, activation of the PI3K/Akt pathway by EGF in prostate cancer cells led to stabilization of HIF-1α and inhibition of PI3K led to decreased transcriptional activity of HIF-1α [108]. Another link between Akt and HIF-1α is provided by data showing that mTOR inhibition decreases HIF-1α stabilization [109]. Similar to *PTEN*, loss of p53 in colorectal cancer cells led to an increase in HIF-1α protein levels and transcriptional activity in hypoxia, possibly due to a decrease in HIF-1α

proteasomal degradation [110]. Angiotensin II, thrombin, and relevant to glioblastoma, platelet-derived growth factors (PDGFs) have also been shown to induce an increase in HIF-1α expression and transcriptional activity in normoxia, possibly by increasing intracellular ROS levels [111]. Supporting the implication of ROS in HIF stabilization in normoxia, another study reported a ROS-dependent and tumor necrosis factor (TNF)-a mediated upregulation of HIF-1α protein expression [112]. Finally, insulin and the insulin-like growth factor have been associated with HIF-1α stabilization [113, 114].

The exact mechanism by which the HIFs are spatially and temporally regulated is not fully understood. While some studies report absence of HIFs from normal tissues in normoxia [115], others report detectable levels of HIF-1α in normoxia in several tissues, including the brain, kidney, heart, liver, and muscles [116]. HIF-1α levels were found to increase in these organs in response to hypoxia [116]. A study on HIF-2α expression reported the lack of HIF-2α protein expression from normal tissues in normoxia, but high stabilization of the protein under hypoxia in several organs, including the brain, heart, lung, kidney, liver, pancreas, and intestine [117]. HIF protein levels were reported to be stabilized quickly after exposure to hypoxia, with the expression returning to baseline levels after 3-9 hours of exposure for HIF-1α [116] and after at least 6 hours of exposure for HIF-2α [117]. These data are in accordance with a more recent study in neuroblastoma that showed acute stabilization of HIF-1α followed by a prolonged accumulation of HIF-2α [118]. Preferential degradation of HIF-1α mediated by interactions of HIF-1α with HAF or the Hsp70/CHIP complex could provide a molecular mechanism for the stabilization of HIF-2α in prolonged hypoxia [104, 119]. Interestingly, specific cell populations within organs have shown higher HIF-2α expression compared to other cell populations, while HIF-1α and HIF-2α were reported to be expressed in different cell types within the same organ [116, 117]. These data support the existence of intra-tissue heterogeneity in hypoxic responses [117, 120]. The heterogeneity of HIF-1α- and HIF-2α-mediated responses is exemplified in clear cell renal cell carcinoma (ccRCC) where HIF-2α is thought to have a tumor promoting and HIF-1α a tumor suppressive role [121, 122]. Finally, in non-small cell lung cancer, increased HIF-2α expression has been associated with tumor progression, but paradoxically inhibition of HIF-2α led to increased tumor growth in the same mouse model of the disease [123]. All these data underline the complexity in the regulation of cellular responses to hypoxia and suggest that care should be taken when interpreting the results of any study on the HIFs.

Cellular adaptations to hypoxia

The HIF factors are promptly stabilized and become active in the presence of a low oxygen environment. In their capacity as transcription factors the HIFs orchestrate

the cellular response to hypoxia by upregulating several genes that are mediating a multitude of adaptations to low oxygen conditions [124].

Hypoxia is relevant in the context of cancer biology as cancer cells frequently outgrow the oxygen supply. The ensuing hypoxia leads to the activation of HIFs and of the transcriptional cascade they initiate. Examples of typical genes that are regulated by HIF activation are presented in Table 2. Collectively, the many genes regulated by the HIFs lead to cellular adaptations related to transcriptional regulation, pH regulation, increased glucose metabolism, amino-acid metabolism, increased angiogenesis, erythropoiesis, increased invasion and metastasis, decreased apoptosis, and increased cell survival and cell proliferation [125]. Interestingly, many of these adaptations overlap with the hallmarks of cancer [5, 6], further supporting the notion that hypoxia is an important feature of cancer biology.

Table 2 Cellular adaptations to hypoxia

HIF activation leads to the transcription of several target genes that organize the cellular response to hypoxia. *BHLH340,* basic helix-loop-helix family member E40 (also known as *DEC1*)*; CA9,* carbonic anhydrase 9*; GLUT1,* glucose transporter 1*; GAPDH,* glyceraldehyde-3-P-dehydrogenase*; TGM2,* transglutaminase 2*; VEGF,* vascular endothelial growth factor*; TGF-β3,* transforming growth factor-β3*; EPO,* erythropoietin*; TGF-α,* transforming growth factor-α*; VIM,* vimentin*; ADM,* adrenomedullin*; CCNG2,* Cyclin G2.

Cellular processes	Genes
Transcriptional regulation	*BHLH340*
pH regulation	*CA9*
Glucose metabolism	*GLUT1, GAPDH*
Amino-acid metabolism	*TGM2*
Angiogenesis	*VEGF, TGF-β3*
Erythropoiesis	*EPO*
Invasion/metastasis	*TGF-α, VIM*
Cell survival/ Cell proliferation	*ADM, TGF-α, CCNG2*

A well-studied effect of hypoxia is the increase in radioresistance. Oxygen is necessary for the fixation of DNA double-strand breaks (DSB) by free radicals [126]. Chronic hypoxia has been shown to inactivate the nonhomologous end joining protein DNA-PK [127] and inhibition of this protein led to increased radiosensitivity of hypoxic cells in non-small cell lung cancer *in vivo* [128].

Glioblastoma microenvironment

For years it was thought that cancers are simply an accumulation of homogenous malignant cells. This reductionist view was abandoned after the realization that cancers are complex systems where cancer cells, which themselves alone show a high degree of heterogeneity, interact with surrounding cells of the tumor microenvironment. Immune cells, fibroblasts, endothelial cells, pericytes, adipocytes, and mesenchymal stem cells are amongst the most studied stromal cell types that are associated with cancer cells in solid tumors [129].

Not only the cellular compartment, but also the extracellular matrix (ECM) is differentially composed in tumors compared to normal tissue [129]. The remodeled ECM in combination with the tumor-associated stromal cells of the tumor microenvironment affect tumor cell properties, generate unique niches for tumor cells and provide novel therapeutic opportunities.

Stromal cells in the glioblastoma microenvironment

The interaction between tumor cells and stromal cells in gliomas has been extensively studied. Astrocytes, pericytes, endothelial cells and immune cells, such as macrophages and microglia, dendritic cells, lymphocytes, and neutrophils, are all present in the brain tumor microenvironment and have been associated with tumor maintenance [130, 131].

Pericytes

Astrocytes, pericytes, and endothelial cells are the main components of the blood-brain barrier (BBB) [132]. Astrocytes and endothelial cells are well-studied in the context of glioblastomas and their role in glioblastoma biology will be discussed in later chapters.

High pericyte coverage has been associated with worse survival of glioblastoma patients after chemotherapy [133], possibly indicating that an intact BBB hinders drug delivery to the brain. It has been found that GSCs generate vascular pericytes and that these GSC-derived pericytes supported tumor growth [134]. Interestingly, targeting these pericytes led to disruption of the blood-tumor barrier, but not of the

BBB and increased drug delivery to gliomas *in vivo* [133]. Increasing the permeability of the BBB is a key factor for successfully targeting glioblastoma. The importance of BBB penetration has been exemplified by studies in medulloblastoma. The WNT subtype of medulloblastoma, that is characterized by absence of a functional BBB, responds to therapy much better compared to the other subtypes, that are characterized by an intact BBB [135].

Macrophages and microglia

Although originally the brain was considered an immune privileged organ, it is now clear that the brain and brain tumors are infiltrated by immune cells. Macrophages are the most abundant immune cells in brain tumors [136]. Both bone-marrow derived macrophages and tissue resident microglia infiltrate brain tumors [137], with macrophages being more abundant compared to microglia [138]. Tumor-associated microglia and macrophages (TAMs) were shown to create a pro-tumorigenic environment in glioblastoma, by secreting low levels of pro-inflammatory cytokines and being unable to activate infiltrating T-cells [139]. This anti-inflammatory phenotype was associated with glioma cell proliferation and was even induced by glioma cell-derived colony-stimulating factor 1 (CSF-1) [140]. Microglia has also been shown to promote glioma cell invasion in an EGFR-dependent manner, in a process that is also dependent on glioma cell-derived CSF-1 and was blocked by inhibiting CSF-1 receptor (CSF-1R) signaling [141]. Another study on the crosstalk between microglia and glioma cells reported that glioma cell-derived TGF-β promotes the activation of microglia, which in turn produce IL-1β and increase glioma cell proliferation [142]. Interestingly, Akkari et al. recently showed that TAMs acquire a pro-tumorigenic gene signature after irradiation of the primary tumor and found that inhibition of CSF-1R signaling combined with radiotherapy improved survival in preclinical mouse models of glioma compared to radiotherapy alone [143]. TAMs are also associated with GSC maintenance. GSCs were shown to induce an immunosuppressive macrophage phenotype [144], partially by secreting periostin [145]. TAMs are, in turn, regulating GSC maintenance and tumorigenicity by secreting pleiotrophin [146].

Neurons

In the past few decades, it has become clear that glioma cells are affected by interactions with neurons. Secretion of neuroligin-3 from neurons was found to promote glioma cell growth and blocking its release led to decreased tumor growth *in vivo* [147, 148]. Neural precursor cell-derived pleiotrophin was shown to increase the invasion of glioma cells towards the subventricular zone [149] Moreover, a direct interaction between glioma cells and neurons was recently unveiled. The formation of synaptic connections between the two cells types was found to increase

the proliferation and invasion potential of cancer cells [150, 151]. Finally, expression of the Notch ligand Jagged1 along white matter tracts was found to modulate GSC invasion along these tracks by increased transcription of *SOX9* and *SOX2* [152].

Astrocytes

Astrocytes are glial cells that represent one of the most abundant cell types of the brain. Several processes branch out of the main cell body, leading to a star-like shape that gives them their name (from the Greek άστρο, astro, 'star' + κύτος, kutos, 'cavity/cell').

Astrocytes have multiple roles in the healthy brain [153]. They are involved in regulating blood flow and the BBB function, in maintaining pH and neurotransmitter homeostasis and as a result in maintaining synapse function, and finally, in providing glucose to the adjacent neurons during hypoglycemia [153].

Astrocytes are not just active in the healthy brain, but they also have several functions in response to central nervous system injury or disease. The astrocytic response is termed reactive astrogliosis and reactive astrocytes undergo molecular, cellular, and functional changes in order to restore normal brain function [153, 154]. One of the hallmarks of reactive astrocytes, is cellular hypertrophy, characterized by an enlargement of the cell body with simultaneous shortening of the cellular processes [155] (Figure 5). Other morphological changes include elongation of the cell body, extension of processes towards the site of injury, and process overlap [155]. The degree to which astrocytes acquire these features depends on the severity of the reactive astrogliosis [153].

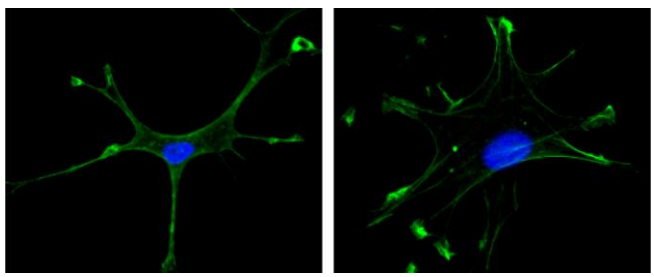

Figure 5 Reactive astrocytes are characterized by somatic hypertrophy
Normal astrocytes (left panel) have long processes that allow them to functionally connect with neurons and pericytes, amongst other cells. Reactive astrocytes (right panel) have enlarged cell bodies and shorter cellular processes. Nuclei in blue, actin in green.

The process of reactive astrogliosis can be initiated as a response to various signaling molecules including growth factors, cytokines such as interleukin 6 (IL-6), TNF-α, interferon (IFN)-γ, and TGF-β, neurotransmitters, ROS, hypoxia, as well

as others [156]. In cases of severe astrogliosis, a glial scar is formed by the astrocytes surrounding the site of injury [153]. These scars provide a venue were astrocytes can interact with other cells, such as inflammatory and fibrotic cells [157], and they assist in astrocyte-mediated regeneration of the nervous system [158]. The formation of the glial scar also allows reactive astrocytes to protect healthy brain cells from inflammatory cells, infectious agents present in the site of injury, and oxidative stress, as well as to facilitate the BBB repair [156].

Defining the markers of reactive astrocytes has been a challenge. Relying on a single marker fails to acknowledge that astrocyte reactivity is a complex phenomenon and that the reactive phenotype manifests differently depending on factors such as the type and extent of the injury [159]. Historically, GFAP expression [160] and somatic hypertrophy [161] have been used as markers of reactive astrocytes. However, recently, a consensus statement was published regarding reactive astrocyte nomenclature and definitions, proposing the use of a combination of molecular markers and functional assays to characterize astrocyte reactivity [159]. The molecular markers that were suggested included increased expression of GFAP, nestin, vimentin, STAT3, and S100B. Functional assays such as calcium and glutamate uptake analysis, glial scar formation, proliferation, and production of ECM, cytokines, and chemokines were amongst the reactivity markers proposed [159].

Astrocytes in the glioblastoma microenvironment

Because astrocytes are an abundant cell type of the glioblastoma microenvironment, several links between them and glioma cells have been established.

Reactive astrocytes are present in the area surrounding glioblastoma lesions [162] and contribute to glioblastoma growth and aggressiveness [163, 164]. Astrocytes express factors such as C-X-C motif chemokine ligand 12 (CXCL12, also known as SDF1) [165], which has been linked to glioma cell growth by interacting with its receptor C-X-C motif chemokine receptor 4 (CXCR4) [166]. Glioblastoma cells were shown to interact with astrocytes via extracellular vesicles and shift astrocytes to a pro-tumorigenic phenotype [167, 168]. Astrocytes and glioma cells also communicate via gap junctions and this communication led to increased glioma cell migration and invasion [169-171], as well as increased resistance to temozolomide [172] and other chemotherapeutic drugs [173]. Tumor-associated astrocytes were shown to express anti-inflammatory cytokines and establish an immunosuppressive glioma microenvironment [174]. Tumor-associated astrocytes of the perivascular niche express osteopontin which was associated with stemness maintenance and enhanced glioblastoma growth in a mouse model of glioma [175]. Astrocyte-conditioned medium was found to contain several proteins associated with invasion and astrocytes were shown to increase the invasive potential of GSCs [176]. All in all, astrocytes are involved in glioma cell and GSC biology.

Brain tumor extracellular matrix

The brain ECM consists mainly of glycosaminoglycans, proteoglycans, and glycoproteins, with the most abundant being hyaluronan, lecticans, link proteins, and tenascins [177]. On the contrary, molecules such as fibronectin and collagens that are abundant in other tissue ECMs, are nearly absent from the brain ECM [177].

The composition of the brain tumor ECM has not been extensively researched but studies show that it is different than that of the normal brain. Tenascin, laminin, fibronectin, and collagen type IV were expressed in an invasive glioblastoma model [178]. Tenascin C is overexpressed in glioblastoma and has been associated with decreased patient survival [179]. It has also been closely linked to glioma angiogenesis, proliferation, and migration [180] and was found to modulate the maintenance of the GSC population by activating Notch signaling [181]. ECM stiffness has also been associated with increased glioma cell proliferation and invasion [182], possibly by leading to activation of EGFR signaling [183]. Interestingly, the increased survival of patients with IDH mutations was attributed to a decrease of tissue stiffness due to a HIF-1α-dependent decrease in tenascin C levels [184]. This study provided a link between the hypoxic microenvironment, ECM stiffness, and glioma aggressiveness.

The role of transglutaminase 2 in tumor biology

Transglutaminases are a superfamily of enzymes that catalyze post-translational protein modifications and crosslink a vast array of proteins in a Ca^{2+}-dependent manner [185].

Transglutaminase 2 (TGM2) belongs in the papain-like family of transglutaminases. It is secreted from the cell to the ECM, where it is involved in ECM organization. TGM2 acts as an integrin co-receptor, in a process independent of its crosslinking activity [186]. It also crosslinks fibronectin, collagen, laminin, and osteopontin, contributing to ECM stabilization and it interacts with growth factors, leading to transcriptional regulation of ECM genes [187]. TGM2 has been reported to translocate to the nucleus, where it is possibly involved in chromatin modifications and gene expression [187].

TGM2 has been associated with more aggressive disease in several cancers such as breast [188] and colorectal [189] cancer, renal cell carcinoma [190], and glioblastoma [191]. In breast cancer, TGM2 expression led to increased cell invasion and survival [192], and was associated with breast cancer cell resistance to chemotherapeutics by activating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling [193].

TGM2 was heterogeneously expressed in a cohort of brain tumor patient samples, with upregulated levels being more frequent in glioblastoma samples and high TGM2 expression being associated with decreased patient survival, regardless of the glioma grade [194]. TGM2 was upregulated in cells expressing high levels of the transmembrane glycoprotein CD44 and was associated with increased cell proliferation and reduced apoptosis [195]. Inhibition of TGM2 resulted in a decrease in fibronectin bundling, and led to increased chemosensitivity of glioma cells *in vitro* and in a mouse model of glioma *in vivo* [196]. Interestingly, TGM2 has also been involved in the maintenance of the GSC population [197] and in increased chemoresistance, a feature of CSCs, of glioma cell lines [194].

HIF signaling in glioblastoma

The role of hypoxia in several aspects of tumor biology was discussed in a previous chapter. In glioblastoma specifically, lesions are characterized by extensive hypoxia.

Interestingly, glioblastomas are highly vascularized tumors [25]. However, glioblastoma cells are highly proliferative and as a result they outgrow the existing vasculature. This leads to the creation of an oxygen gradient from the blood vessels to progressively more hypoxic regions, and finally the necrotic core. Oxygen concentrations ranging between 2-5% and 0.5% (mild hypoxia) and between 0.5% and 0.1% (severe hypoxia) have been measured in glioblastoma lesions [73, 74]. This feature of highly oxygenated, perivascular regions being in close proximity to hypoxic or necrotic regions creates a unique environment for glioma cells and stromal cells present in the glioma microenvironment.

In the hypoxic regions, the lack of oxygen leads to the stabilization of the HIFs and the initiation of cellular responses to hypoxia. HIF stabilization can also occur as a result of specific pathway alterations. Glioblastomas harbor several genetic aberrations, some of which were discussed previously [27, 31] and several of which can lead to HIF stabilization. Loss of p53 function [110], loss of PTEN [107, 108], PI3K-Akt signaling [108], EGFR signaling [108], and loss of ARF function [198] are some of the aberrations present in glioblastoma that can lead to increased HIF-1α activity.

Hypoxia signaling greatly affects glioblastoma biology and has a tumor-promoting effect. For instance, HIF-1α-induced upregulation of CXCR4, the receptor for CXCL12, was found to be involved in glioma cell migration [199]. HIF-1α-dependent increase of CXCL12 and VEGF also led to the upregulation of glioma cell invasion and increased angiogenesis [200]. These studies suggest that the infiltrative nature of glioblastomas can be partially explained by activated HIF-1α signaling. Moreover, HIF signaling has been associated with worse overall survival

of glioblastoma patients [201]. Finally, hypoxia is a crucial factor in the maintenance of the GSC population [202, 203].

Glioblastoma stem-like cell niches

The existence of GSC and their role in glioblastoma biology has been extensively studied [204]. Glioma cells are characterized by a high degree of plasticity between stem- and non-stem-like states [38] and part of this phenotype switch is regulated by microenvironmental cues, such as hypoxia [39]. Interestingly, GSCs are enriched in the oxygenated perivascular niche and the hypoxic perinecrotic niche [56].

The hypoxic environment of the perinecrotic niche is a well-studied contributor of glioma stemness [202, 203]. Broadly used stemness markers such as Nanog and Oct4 are upregulated by hypoxia in glioblastoma cell lines [205] and neurospheres [206]. HIF signaling is important for the maintenance of GSCs [207, 208]. HIF-1α mediated the dedifferentiation of glioma cells to GSCs and is a crucial factor for GSC maintenance by activating the Notch pathway [209, 210]. HIF-2α leads to increased glioma cell stemness by regulating the transcription factors Oct4 and SOX2 [211].

There are several studies supporting the role of the perivascular niche in GSC maintenance. GSCs were found in close proximity to blood vessels in glioblastoma, with endothelial cells being crucial in maintaining this cell population [212]. Endothelial cells have also been reported to maintain the GSCs by secreting Notch ligands [213] and leading to mTOR pathway activation [214]. Conversely, GSCs are known to interact closely with endothelial cells. GSCs produce high levels of VEGF and exhibit angiogenic properties [215]. Moreover, there is evidence that GSCs differentiate into endothelial cells [216-218] and pericytes [134], however these studies should be interpreted with caution, as the use of single markers to define GSCs, endothelial cells, or pericytes can affect the results. Interestingly, HIF-2α was stabilized in GSCs at physiological oxygen concentrations (5% oxygen, pseudo-hypoxia) [207], indicating that even within the well-oxygenated environment of the perivascular niche, HIF signaling regulates glioma stemness.

CD44 signaling in glioblastoma

CD44 structure, cleavage, and function

CD44 is a type I transmembrane glycoprotein involved in cell-cell and cell-matrix interactions. It is encoded by a single *CD44* gene and characterized by the existence

of various isoforms. The isoforms are generated by alternative RNA splicing and various posttranslational modifications, such as glycosylation or the addition of glycosaminoglycans. The most common isoform is the CD44 standard which contains 7, 1, and 2 exons encoding the extracellular, the transmembrane, and the intracellular domain, respectively. The CD44 variant isoforms (CD44v) are generated by the addition of up to 11 alternatively spliced exons (v1-v11) in a specific site of the extracellular domain, between exons 5 and 6 [219, 220]. The intracellular domain is also subject to alternative splicing, leading to a short or long cytoplasmic tail, with the latter being more common. The extracellular and intracellular domains are highly conserved and show 80-90% homology between species [219].

CD44 acts primarily by binding to its main ligand, the ECM glycosaminoglycan hyaluronan (HA) [221]. Other ECM components, such as collagen, laminin, and fibronectin [222] or osteopontin [223] also act as ligands for CD44. The signal transduction mediated by CD44 is regulated by its cleavage. The extracellular domain of CD44 is cleaved by membrane-associated metalloproteases (MMPs), such as membrane type-1 MMP (MT1-MMP) [224], and a disintegrin and metalloproteinase (ADAM)-like proteases, such as ADAM10 and ADAM17 [225, 226]. A second cleavage of the intracellular domain is mediated by γ-secretase and leads to the release of the intracellular domain (ICD) fragment [227]. The CD44ICD translocates to the nucleus, interacts with the transcriptional coactivator CBP/p300 and activates a transcriptional program of genes harboring the 12-*O*-tetradecanoylphorbol 13-acetate-responsive element (TRE) [227] (Figure 6).

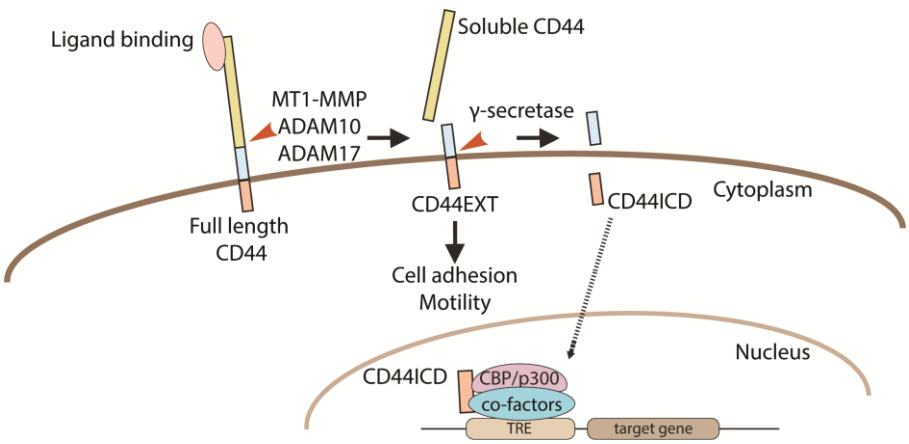

Figure 6 CD44 stucture and cleavage
The full length CD44 undergoes two sequential proteolytic cleavages, one mediated by MT1-MMP, ADAM10 or ADAM17 and the second by γ-secretase. The product of the second cleavage, the CD44ICD, translocates to the nucleus where it interacts with transcriptional co-activators, such as CBP/p300, and transcription factors. It binds to TREs of target genes, and regulates their transcription.

CD44 signaling has been implicated in several processes. These depend on the cellular context and the isoform expressed in each cell type. During embryonic development, CD44 is expressed both pre- and post-implantation [228, 229] and is associated with embryonic neuronal development [230]. In adults, the various CD44 isoforms are expressed primarily by epithelial cells of several tissues [231]. CD44 activation leads to fibroblast proliferation through PDGF-BB signaling [232] and fibroblast migration through TGF-β activation [233]. CD44 and HA interactions are also important during wound healing [234].

Since CD44 is involved in cell proliferation and migration, CD44 signaling also has implications in tumor biology. Notably, proteolytic cleavage of CD44 and generation of the CD44ICD is frequent in glioma, breast cancer, non-small cell lung cancer, colon and ovarian cancer, among others [235].

Expression of the CD44 standard isoform in pancreatic cancer was associated with increased tumor growth, with the ensuing tumors expressing CD44 variant isoforms, suggesting an isoform switch [236]. CD44 cleavage also induced migration of pancreatic cells *in vitro* [237] and increased migration of breast cancer cells *in vivo* [238]. Inhibition of the CD44v6 isoform signaling in a pancreatic cancer xenograft model led to decreased tumor growth and metastasis [239]. In a colon cancer cell line CD44 expression protected cancer cells from apoptosis [240]. Blocking of CD44 signaling abolished endothelial cell proliferation and migration *in vitro*, suggesting that active CD44 is involved in angiogenesis [241].

The CD44 standard was the main isoform identified in human glioblastoma samples [242]. Inhibition of CD44 signaling inhibited the migration of glioblastoma cells *in vitro* [243]. CD44-induced glioma cell invasion and proliferation might be mediated in an EGFR-dependent manner, by direct interaction of the CD44 extracellular domain with EGFR [244]. CD44 signaling has also been implicated in therapy resistance, by inhibiting the Hippo pathway activation and protecting glioma cells from apoptosis [245]. Finally, CD44 expression correlated with worse patient survival in the proneural subtype of glioblastoma [175].

Role of CD44 in cancer stemness

CD44 is highly associated with cancer stemness. CD44 expressing cells with stem-like properties have been identified in breast, pancreatic, and colon cancer, as well as in leukemia and glioblastoma [48, 49, 246-248].

In breast cancer, various CD44 isoforms were present and correlated with breast cancer subtypes [249]. Breast cancer cells expressing high levels of CD44 also expressed high levels of HA and exhibited features of epithelial-to-mesenchymal transition, such as activated TGF-β signaling and expression of Snail and Twist [250]. This suggests that CD44/HA signaling is implicated in breast CSC maintenance. CD44 expressing pancreatic cancer cells have been associated with

chemotherapy and radiotherapy resistance [251, 252], both features of CSCs. Interestingly, targeting CD44-positive pancreatic CSCs by inducing KLF4 expression led to a decrease in stemness features *in vitro* and of metastasis *in vivo*, indicating that targeting CD44 is a potential therapeutic target [253]. Finally, CD44v6 was expressed in colorectal CSCs and was associated with their increased metastatic capacity [248].

In glioblastoma, CD44 was one of the first GSC markers used for the isolation of GSCs, proposed along with CD133 [51, 246]. CD44 was shown to promote stem-like cell properties, such as stem cell marker expression, radiation resistance, and sphere formation of glioblastoma cells [254]. Moreover, it has been shown that CD44 and its ligand osteopontin were both expressed in the perivascular niche of a murine model of glioma [175]. Interestingly, binding of CD44 to osteopontin led to an increase in stemness features such as drug efflux and stem cell marker expression in a manner dependent on the cleavage of CD44 by γ-secretase and the generation of CD44ICD. Expression of the CD44ICD by glioma cells led to an increase in stemness features such as stem cell marker expression, drug efflux, and radiation resistance. These phenotypes were shown to be a result of increased stabilization of HIF-2α by an interaction between CBP/p300 with CD44ICD [175]. These data provide a link between CD44 signaling, hypoxic signaling, and stemness in glioblastoma.

$p75^{NTR}$ signaling in glioblastoma

Structure and signal transduction

Neurotrophins, such as the nerve growth factor (NGF) and the brain-derived neurotrophic factor (BDNF), are a family of growth factors regulating the development and maintenance of the nervous system [255, 256]. The p75 neurotrophin receptor ($p75^{NTR}$) is one of the two receptor types mediating signaling via the neurotrophins, the second being the tropomyosin-related kinase (Trk) tyrosine kinase receptor family [255, 256]. $p75^{NTR}$ acts as a receptor for all neurotrophins and as a co-receptor for Trk receptors.

The $p75^{NTR}$ receptor is a type I transmembrane protein. The extracellular domain consists of four cysteine-rich repeats and the intracellular domain contains a death domain [256]. It belongs to the TNF receptor family and as such interacts with several downstream signaling molecules such as Schwann cell 1, receptor-interacting protein 2, RhoA, Jun N-terminal kinase, and NF-κB [256]. In physiological conditions or in response to nervous system injury signaling via the $p75^{NTR}$ receptor can lead to pro-apoptotic or pro-survival signals depending on the cellular context [256].

The $p75^{NTR}$ receptor is, similar to CD44 and other transmembrane proteins, such as Notch, subjected to sequential proteolytic cleavages. Metalloproteases, such as ADAM17, cleave the full-length receptor and generate a soluble extracellular fragment and a membrane bound fragment containing the transmembrane domain and the ICD (carboxyterminal fragment, CTF) [257, 258]. The membrane bound fragment is subsequently cleaved by γ-secretase, leading to the release of the p75ICD [257, 259] and its translocation to the nucleus [260]. Interestingly, γ-secretase cleavage of $p75^{NTR}$ was shown to be induced by hypoxia and led to the stabilization of HIF-1α [261], providing a direct link between $p75^{NTR}$ signaling and hypoxia.

Role of $p75^{NTR}$ in glioblastoma biology

The $p75^{NTR}$ receptor is well studied in the context of tumor biology. NGF-dependent activation of $p75^{NTR}$ was shown to increase the survival of breast cancer cells [262], while inhibition of $p75^{NTR}$ expression induced cell death of esophageal squamous cell carcinoma cell lines [263]. $p75^{NTR}$ activation increased cell survival and migration in ccRCC cell lines [264] and signaling mediated by $p75^{NTR}$ has been associated with increased migration and epithelial to mesenchymal transition in melanoma cells [265, 266]. Finally, the $p75^{NTR}$ receptor was upregulated in lung and thyroid cancer [267, 268].

Furthermore, $p75^{NTR}$ signaling is associated with stemness in several cancers. $p75^{NTR}$ expressing cells with stem-like properties have been identified in melanoma [269], esophageal and squamous cell carcinoma [270, 271], breast cancer [272], and hypopharyngeal cancer [273]. However, the exact mechanism underlying the regulation of stemness features by $p75^{NTR}$ is unknown.

In glioblastoma, expression of $p75^{NTR}$ is well-documented and has been linked to migration and invasion. Activation of $p75^{NTR}$ by NGF led to increased migration and invasion of glioma cells *in vitro* and $p75^{NTR}$ overexpression led to increased invasion *in vivo* [274], in a γ-secretase-dependent and -independent manner [275, 276]. $p75^{NTR}$-mediated glioma cell invasion is dependent on interaction of $p75^{NTR}$ with PDLIM1, a protein involved in invasion in other tumor types [277].

Emerging therapies

Despite rigorous research, the improved understanding of the molecular characteristics of glioblastoma has not translated to novel therapies. As a result, treatment options available for glioblastoma patients have not changed much over the past several decades. The addition of concomitant (together with radiotherapy) and adjuvant (after radiotherapy) temozolomide significantly improved the 5-year

survival of patients, with 9.8% of the patients reaching the 5-year follow-up compared to only 1.9% of those treated with radiotherapy alone [9]. In the early 2000s, the addition of 5-aminolevulinic acid for fluorescence-guided maximal surgical resection improved progression-free survival after surgery by 6 months compared to white light-guided resection [10]. Finally, more recently, the addition of tumor-treating fields along with maintenance temozolomide extended the progression-free survival of glioblastoma patients by nearly 3 months [12].

Despite all these treatment options, however, the median survival for glioblastoma patients is only 8 months after diagnosis, making this the lowest median survival among primary malignant brain and other central nervous system tumors [7]. It is therefore crucial to develop new therapies for these patients. Over the years, several therapeutic targets have been proposed, with some therapies reaching clinical trials. Unfortunately, a recent study found that only 9% of phase III clinical trials on primary or recurrent glioblastoma lead to an improvement in overall survival, despite them being based on successful phase II clinical trials [278]. The authors also noted that several, eventually failed, phase III trials were initiated after failed phase II trials, or without any phase II trials having been conducted [278]. The inability of potential therapeutics to lead to improved patient outcomes can be partially attributed to the inability of many drugs to cross the BBB, the lack of druggable targets in glioblastoma, the existence of redundancy in signaling pathways, the high degree of intratumoral heterogeneity, and the frequent development of therapy resistance.

In this chapter, various aspects of the glioma microenvironment and how they affect the aggressiveness of glioblastoma have been presented. It is therefore logical that new approaches need to target not just the glioma cells but also the microenvironment in order to lead to successful treatment of glioblastoma patients.

Glioblastomas are highly vascularized tumors [279] and as such, anti-angiogenic therapies were predicted to improve patient outcomes. Bevacizumab, a monoclonal antibody blocking VEGF function, was assessed in glioblastoma along with temozolomide and radiotherapy (NCT00943826). Even though treatment with bevacizumab increased progression-free survival, it did not lead to an increase in overall survival and was accompanied by an increase in adverse effects [280]. Due to the increase in progression-free survival, bevacizumab was included in the standard care of recurrent or progressing glioblastoma in the United States, but it has not been approved for use in Europe.

Promising preclinical data suggested that constitutive activation of EGFR signaling due to the EGFRvIII led to increased tumor aggressiveness. This led to the generation of inhibitors such as erlotinib, antibodies, and vaccines such as rindopepimut, targeting this mutated receptor. Despite some promising phase II trials, targeting EGFRvIII did not ultimately lead to successful phase III trials, measured by an increased overall survival [30]. Interestingly, a phase I clinical trial

on recurrent glioblastoma using chimeric antigen receptor T cells targeting the EGFRvIII, found that after treatment, there was a marked decrease of EGFRvIII expression in the recurrent tumors [281]. This observation suggests that even if EGFRvIII targeting proves successful in a primary setting, it might prove futile in recurrent glioblastoma due to survival and expansion of clones that do not express the mutated receptor.

One aspect of the microenvironment that could be targeted is the interaction between astrocytes and glioma cells. Astrocytes interact via gap junctions with glioma cells [169]. This interaction via the C-terminus of connexin 43 (Cx43) [169] could be used to guide cisplatin-loaded nanoparticles to tumor cells [282], thus achieving specific tumor cell killing and decreasing the adverse effects of chemotherapy. Astrocytes of the tumor microenvironment have also been found to produce CXCL12 which interacts with its receptor CXCR4 [165, 166]. A CXCL12 inhibitor was shown to enhance bevacizumab treatment efficacy in a rodent model of glioma [283], while a CXCR4 antagonist was found to decrease astrogliosis and vasculogenesis *in vivo* [284]. These studies suggest that anti-angiogenic therapy can be improved by inhibiting stromal astrocyte-glioma cell interactions, although no such clinical trials have been performed.

Another aspect of the tumor microenvironment that could be targeted is the ECM. The ECM of brain tumors differs from that of the normal brain. As a result, components of the tumor ECM can be used as therapeutic targets. Tenascin C was used to guide nanoparticles to glioma lesions in a mouse model [285] and antibodies against tenascin C were shown to specifically target tumors in a rat model of glioma [286], suggesting that these can be used for targeted drug delivery to gliomas. Moreover, inhibition of TGM2 led to increased sensitivity of an *in vivo* model of glioma to the chemotherapeutic nitrosourea [196]. These studies support the hypothesis that targeting the ECM could improve patient responses to treatments and could provide the basis for future clinical trials.

Hypoxia is another hallmark of glioblastoma biology. Targeting hypoxia would not only decrease the migratory phenotype of the hypoxic glioma cells but also attenuate glioblastoma stemness. Several HIF inhibitors have been tested in clinical trials for various cancers [287]. Vorinostat, a histone deacetylase inhibitor that increases HIFα degradation [287], was used as a single agent in a phase II study of recurrent glioblastoma as well as in combination with temozolomide and radiation therapy in a phase I/II trial for newly diagnosed glioblastoma (NCT00731731) [288, 289]. In both cases the inhibitor was well tolerated but only had minimal effects in progression free survival. PT2385 is a HIF-2α antagonist that inhibits the dimerization of the HIFα and HIFβ subunits and therefore the binding to the DNA [290]. This HIF-2α inhibitor has been tested in a phase II clinical trial for recurrent glioblastoma but showed only minimal improvement of progression-free survival. A second-generation inhibitor, PT2977, was used in a clinical trial of patients with advanced solid tumors, including recurrent glioblastoma (NCT02974738);

however, the trial has reported promising results only in ccRCC patients thus far [291].

Another feature of glioblastomas is cancer stemness. Targeting cancer stemness would lead to the eradication of the slow proliferating, chemotherapy and radiotherapy resistant cells that give rise to recurrences. The resistance of GSCs to radiation therapy is well established and heavily depends on DNA damage response (DDR) pathways [292]. Inhibition of these pathways could improve the response of these cells to radiotherapy. For example, the radiotherapy resistant phenotype of GSCs was reduced by inhibition of ataxia telangiectasia mutated (ATM), a protein of the DDR pathway that regulates DSB repair and cell cycle checkpoints [293]. The specific ATM inhibitor KU-60019 sensitized glioma cells to irradiation *in vitro* and intracranial administration of the inhibitor via osmotic pumps was well tolerated by healthy mice *in vivo* [294, 295]. Another way to target GSCs is by restoring p53 signaling. A nanoparticle delivering the *TP53* gene, which is frequently deleted in glioblastoma [27], targeted the CD133 positive glioma cells [296], a population that is to a large degree comprised of GSCs [51]. It also decreased the resistance to temozolomide both *in vitro* and *in vivo* [297], leading to improved survival in a mouse model of glioma. The same *TP53* gene delivery system was tested in combination with temozolomide for the treatment of recurrent glioblastoma, but the results of the clinical trial (NCT02340156) have yet to be published.

ADAM- and γ-secretase-dependent cleavage of proteins such as CD44 and $p75^{NTR}$ contributes to glioblastoma aggressiveness. Therefore, targeting this cleavage could improve patient outcomes. ADAM10 and ADAM17 inhibition by a broad-spectrum inhibitor of MMPs and ADAMs, TAPI-2, has been shown to promote differentiation of GSCs [298] and to reduce GSC growth by inhibiting Notch [299]. Several studies and clinical trials have examined the role of γ-secretase inhibitors in glioblastoma, mainly evaluating the effect of the drugs on Notch signaling [300] but the results might be mediated by the γ-secretase inhibitors also blocking CD44 or $p75^{NTR}$ cleavage and signaling. Inhibition of γ-secretase activity by the γ-secretase inhibitor DAPT decreased invasion and increased survival in a mouse model of glioma by blocking $p75^{NTR}$ signaling [275]. Moreover, another γ-secretase inhibitor, MRK003, decreased the viability and sphere-formation ability of glioma cells with high expression of CD44 *in vitro* [301]. However, a phase II clinical trial (NCT01122901) using the γ-secretase inhibitors RO4929097 or DAPT showed minimal progression-free and overall survival benefit for patients with recurrent glioblastoma [302]. A second phase II trial (NCT03422679) including glioblastoma is ongoing.

From this brief presentation of available and currently tested therapies it is clear that new therapeutic approaches are needed for the treatment of glioblastoma, one of the most aggressive tumor types. This thesis aims to assist in the efforts to identify new therapeutic targets by improving our understanding of glioblastoma cells and their interactions with the glioma microenvironment.

The present investigation

Overview and aims

Glioblastoma is the most aggressive primary brain tumor in adults. Despite new treatments being tested frequently this disease remains largely untreatable, with only about 7 out of 100 patients diagnosed with glioblastoma surviving for 5 years. This indicates an unmet need for new therapies. With this goal in mind, this thesis aims at improving our understanding of glioblastoma biology and identifying new therapeutic opportunities. More specifically we set out to study the effect of treatments and of hypoxia on tumor-associated astrocytes (Papers I and II), and the role of hypoxic signaling in the maintenance of the GSC population (Papers III and IV).

Paper I: The irradiated brain microenvironment supports glioma stemness and survival via astrocyte-derived transglutaminase 2

In this study, we aimed to investigate the effect of irradiation on stromal cells in the context of glioblastoma. We showed that astrocytes become reactive both *in vitro* and *in vivo* in response to radiation treatment. Reactive astrocytes secrete TGM2 in the ECM, in a process mediated by TNF-α. TGM2 in turn promotes stemness features in glioma cells, including drug efflux and radioresistance, by activating integrin/Src signaling. All in all, we showed that irradiation of the primary tumor affects stromal astrocytes in a manner that primes the microenvironment for further treatment resistance, and we propose TGM2 as a potential new therapeutic target for glioblastoma.

Discussion

Glioblastomas are characterized by the presence of therapy resistant cells, the GSCs. GSCs are resistant to both chemotherapy [303], and radiation therapy [64], and as a result they have been implicated in disease progression and tumor recurrence.

Interestingly, several studies in recent years have unequivocally proven that the GSC state is not rigid. Instead there is a high degree of plasticity between GSCs and bulk tumor cells, with one population giving rise to the other depending on intrinsic genetic factors and extrinsic properties of the tumor microenvironment [37-39, 304]. The factors that determine the interconversion between these phenotypic states are poorly understood. Interestingly, a study on disseminated breast cancer cells, found that factors derived by bone marrow-resident mesenchymal stem cells induce breast cancer cell dormancy, providing one explanation for the generation of metastasis several years after the treatment of the primary tumor [305]. This study underlines the role of the microenvironment in maintaining stemness. Identifying the factors that lead to increased glioma stemness and consequentially treatment resistance can provide insights into novel therapeutic targets for glioblastoma.

Radiation therapy is part of the standard of care for glioblastoma patients [9]. Interestingly, irradiation of the brain led to increased infiltration of the healthy brain by glioma cells in a rat model of glioma [306]. Moreover, irradiation increased the invasiveness of glioma cells [307-310], possibly by activating Src/Rho signaling [308, 309]. It has also been proposed that the glioma microenvironment supports glioma cell radioresistance after observations that glioma cells of an orthotopic model of glioma were better at activating the DSB repair mechanism *in vivo* compared to their *in vitro* cultured counterparts [311]. However, there is little research showing the effect of ionizing radiation on stromal cells of the glioma microenvironment. This study proposes a mechanism though which radiation-induced astrocyte reactivity affects glioma cell stemness in a TGM2-dependent manner.

Activation of TGM2 is regulated by several factors. IL-6 [312], TGF-β [313], and TNF-α [197] are amongst the pro-inflammatory cytokines that have been associated with increased TGM2 expression, while oxidative stress and radiation treatment have also been implicated in TGM2 activation [314]. We found that in our cell system, addition of exogenous TNF-α led to an increase in TGM2 expression in cultured primary astrocytes. This however does not exclude the possibility of other factors affecting TGM2 deposition in the ECM of glioblastomas.

In this study we saw that irradiated and reactive astrocytes secrete TGM2 in the ECM. Several studies have shown that inhibition of TGM2 in glioma cells abrogates their stemness features [191, 195-197]. Here, we show that increased TGM2 secretion in the ECM affects glioma cell stemness by activating the integrin/Src pathway. Inhibition of Src signaling has been shown to inhibit GSC migration, metabolic plasticity, and survival [315-317]. Recently, it was shown that Src inhibition increases the sensitivity of GSCs to radiation by inhibiting the EGFR/PI3K/Akt signaling pathway [318]. Importantly, the addition of a Src inhibitor along with radiation therapy prolonged the survival of mice in an orthotopic xenograft glioma model, compared to radiation alone [318]. Moreover, integrin a7 signaling has been shown to increase GSC growth and invasiveness

[319]. These studies support our proposed mechanism of GSC regulation by TGM2, via integrin. Unfortunately, cilengitide, an integrin αvβ3 and αvβ5 inhibitor, did not improve outcomes when combined with temozolomide in a phase III clinical trial of primary glioblastoma patients with methylated *MGMT* promoter [320] or combined with radiotherapy and temozolomide treatment in a phase II clinical trial of primary glioblastoma patients with unmethylated *MGMT* promoter [321]. However, targeting the integrin pathway might still prove effective in combination with radiation treatment in patients with *MGMT* promoter methylation.

Based on our findings, therapeutic targeting of TGM2 in combination with radiation therapy could minimize the tumor promoting effects of irradiated astrocytes and improve patient outcomes. In this study, we used two TGM2 inhibitors; GK921 and dansylcadaverine. GK921 is a compound that interacts with the N-terminus of TGM2 and leads to a conformational change that inactivates the enzyme [322]. Dansylcadaverine is a general transglutaminase inhibitor that is also used in evaluating transglutaminase enzymatic activity [323]. Recently, an irreversible inhibitor for TGM2 was described [324, 325]. The generation of TGM2 inhibitors that cross the BBB will contribute in its evaluation as a therapeutic target in glioblastoma.

To test the effect of TGM2 inhibition in a model that does not depend on penetration across the BBB, we decided to target TGM2 in *ex vivo* organotypic slice cultures generated from our PDGF-B- and shp53- expressing murine gliomas. This platform has gained traction amongst cancer researchers, with several studies being published using organotypic slice cultures in glioblastoma [174, 317, 326]. Organotypic slice cultures allow for experimental testing of therapeutically targeting novel proteins or pathways in primary brain tumor tissue, without having to rely on the existence of drugs that cross the BBB. Moreover, organotypic slice cultures recapitulate the tumor microenvironment more closely compared to co-culture experiments that are comprised of just a few cell types. In our lab, we have successfully cultured brain slices from tumor-naïve and tumor-bearing mice. We found that tumor slices survive for at least 14 days in culture at 21%, 5%, or 1% oxygen tension, and astrocytes are attracted to the tumor areas in all oxygen tensions (unpublished data). Using Olig2 to differentiate between malignant and stromal cells [327], and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to differentiate between apoptotic and non-apoptotic cells, we have seen that for up to 7 days in culture at 21% oxygen almost 95% of tumor cells and around 60% of stromal cells are alive in the slices (unpublished data). This suggests that when testing the TGM2 inhibitor GK921 in tumor slices in this study, astrocytes were present in the slices in proximity of tumor cells. Moreover, the astrocytes would have the potential to produce ECM in the slices, and notably, TGM2 was detectable in the tumor slices (unpublished data).

The initial screen of our study was based on the side population assay. This assay assesses stemness based on the ability of GSCs to exclude the Hoechst dye due to

the expression of the ABCG2 transporter [66, 328]. However, simple factors such as cell density and Hoechst concentration can affect the outcome of this sensitive assay [329]. To evaluate stemness with more precision, a combination of functional assays can be used. In this study we used the side population assay in combination with the clonal survival following radiation assay (radiation resistance assay), the primary and secondary sphere formation, and the limiting dilution self-renewal assay. All these assays showed an increase in stemness properties of glioma cells cultured on ECM from irradiated astrocytes or cultured in the presence of exogenous TGM2. Collectively these data support the conclusion that radiation-induced TGM2 present in the ECM promotes stemness in glioblastoma. The use of other functional assays could assist in evaluating the contribution of other irradiated stromal cell types in stemness maintenance and could provide more insights into the possibilities of targeting other tumor-promoting stromal cells.

Also, in the initial screen of our study, we used several stromal cell lines in co-cultures with glioma cells derived from a PDGF-B-induced mouse model of glioma. While the endothelial cells used for the screen were murine, the pericytes, microglia and astrocytes were human cell lines. This could result in problems with cross-reactivity, and could indicate a failure to detect effects mediated by the other cell types due to the inability of the human stromal cells to cross-talk with the murine glioma cells. It is therefore possible that irradiation of other stromal cells also affects glioma cell stemness and this remains to be studied further.

Throughout this study we mostly used a single dose of 10 Gy for the irradiation of the astrocytes *in vitro*. We selected this radiation dose based on it being the therapeutic dose in our mouse model of PDGF-B-induced glioblastoma [330]. This radiation dose proved sufficient to show that astrocytes become reactive both *in vitro* and *in vivo* after radiation, that the pre-irradiated tumor-naïve brains support tumor growth, that irradiated astrocytes persist after irradiation in the area where the tumor was, and that they secrete TGM2 *in vivo*. Since patients with glioblastoma receive fractionated radiation in the form of 30 fractions of 2 Gy [9], we evaluated the effect of fractionated doses in addition to the single dose of 10 Gy. More specifically, we confirmed that astrocytes become reactive after 3 fractions of 2 Gy and that TGM2 expression was elevated in recurrent tumors where mice were treated with 5 fractions of 2 Gy, further supporting that our findings are relevant for glioblastoma patients. Interestingly, single-dose ultra-high dose radiation, termed FLASH radiotherapy, has been shown to inhibit tumor growth while also reducing the detrimental effects of radiation in cognitive abilities in a mouse model of glioblastoma [331]. Whether this irradiation regimen also decreases the astrocyte reactivity associated with irradiation remains to be studied.

In vitro experiments with irradiated astrocytes showed that they secrete elevated levels of IL-6 and IL-8 for at least 10 days in culture. The production of ECM from irradiated astrocytes over the course of 8-10 days also showed that at least TGM2 in the matrix was stable until that time point. Moreover, we showed that TGM2

expression is high in recurrent tumors in mice and in a small cohort of glioblastoma patients, suggesting that increased TGM2 signaling might be initiated during the treatment of the primary tumor, but could remain active even at recurrence. All these data suggest that this study has implications not just for primary glioblastomas but also for recurrent tumors.

In conclusion, using a combination of *in vitro*, *ex vivo*, and *in vivo* models we showed that astrocytes become reactive in response to irradiation, they secrete TGM2 in the ECM and affect glioma cell stemness. Therefore, targeting TGM2 activation or its downstream signaling could represent a new therapeutic strategy for glioblastoma.

Paper II: Hypoxia-induced reactivity of tumor-associated astrocytes affects glioma cell properties

In this study we aimed to investigate the response of astrocytes to hypoxia in the context of glioblastoma. We used primary human astrocytes and a PDGF-B- and shp53-induced mouse model of glioma to evaluate the effect of intermediate and severe hypoxia, as well as of physiological oxygen levels, on this stromal cell type. We showed that astrocytes cultured *in vitro* in intermediate (1% oxygen) or severe (0.1% oxygen) hypoxia, but not in physoxia (5% oxygen), undergo reactive astrogliosis, stabilize the HIF factors, and upregulate the expression of several hypoxia-induced genes. Interestingly, astrocytes exposed to long-term severe hypoxia showed higher stabilization of HIF-2α compared to HIF-1α protein levels. Hypoxic astrocytes upregulated the expression of some hypoxia-related cytokines *in vitro* and were found in close proximity to HIF-2α expressing cells *in vivo*. Finally, matrix from hypoxic astrocytes increased the colony formation or the area of colonies, and drug efflux ability of glioma cells grown on it. All in all, we showed that hypoxic astrocytes become reactive and alter the properties of glioma cells in ways that could induce features of stemness and more aggressive tumors.

Discussion

Hypoxia is a hallmark of glioblastoma, with oxygen tensions in the tumor ranging from 0.1% to 5% oxygen [73, 74]. The effect of hypoxia on glioma cells is well-studied. Hypoxia induces glioma cell migration and invasion, and tumor angiogenesis [199, 200]. Importantly and relevant to therapy resistance, hypoxia enhances the stemness phenotype of GSCs [207, 208].

Despite extensive research in the role of hypoxia on glioma cells and GSCs, little is known about the effect of hypoxia on stromal cells of the tumor microenvironment.

Hypoxia has been shown to shift macrophages towards a pro-tumorigenic phenotype *in vitro* [332]. Microglia cultured under hypoxic conditions showed an increase in CXCR4 levels and increased migration *in vitro,* possibly mediated by CXCL12/CXCR4 interaction [333]. Moreover, cross-talk between hypoxic glioma cells and stromal cells induced angiogenesis and pericyte migration *in vitro* [334].

Astrocytes are abundant in the brain tumor microenvironment, yet their response to tumor hypoxia has not been extensively studied [335]. The response of astrocytes to hypoxia has been studied more in the context of ischemic injury, where it has been shown that astrocytes exposed to hypoxia become reactive, form a glial scar, and assume a neuroprotective role [336]. In paper I, we showed that astrocytes respond to irradiation by becoming reactive and that irradiated astrocytes affect glioma cell stemness by secreting TGM2 in the ECM. Based on these observations, we decided to evaluate the effect of hypoxia on astrocytes in the context of glioblastoma and to assess whether hypoxic astrocytes affect properties of glioma cells.

In this study we used three different oxygen tensions representing the physiological oxygen levels in the normal brain (physoxia, 5% oxygen) and the intermediate (1% oxygen) and severe (0.1% oxygen) hypoxia, that are characteristic of the tumor brain. We found that astrocytes exposed to physoxia do not acquire features of reactive astrogliosis nor do they stabilize the HIF proteins. Moreover, physoxic astrocytes do not change the expression levels of a panel of cytokines (unpublished data). These data further support the view that 5% oxygen represents a physiological oxygen tension in the brain [73, 74] and indicate that astrocytes require lower oxygen tensions to activate the hypoxia response pathway.

Interestingly, astrocytes exposed to long-term severe hypoxia preferentially stabilized HIF-2α. This result can be explained in multiple ways involving temporal regulation of HIFα subunit expression, or oxygen-dependent modification of HIF stability. Temporal differences in HIFα stability have been previously reported [118, 337]. In neuroblastoma, HIF-1α and HIF-2α were shown to be temporally regulated by hypoxia, with HIF-2α mediating prolonged responses to hypoxia and being associated with poor prognosis [118]. Differences in the activity of HIF-1α and HIF-2α are seen in processes outside of hypoxia, as well, such as in their roles in cell pluripotency. In the process of reprogramming cells to pluripotency, HIF-1α and HIF-2α were found to both be necessary but to exert different functions depending on their temporal expression [338]. Moreover, HAF, Hsp70, and CHIP have been implicated in the degradation of HIF-1α in prolonged hypoxia [103, 104], and their expression by prolonged exposure of astrocytes to severe hypoxia could explain the preferential stabilization of HIF-2α in these cells. Alternatively, the preferential stabilization of HIF-2α by severely hypoxic astrocytes could be due to the differential stabilization of HIFα subunits at this oxygen tension. As mentioned in a previous chapter, the PHDs are largely responsible for the proteasomal-dependent degradation of the HIFα subunits. The PHDs are themselves regulated

by the E3 ubiquitin ligase Siah2, which leads to a decrease in PHD1 and PHD3 levels in hypoxia [102]. Notably, PHD1 and PHD3 show a preference for binding HIF-2α, whereas PHD2 hydroxylates primarily HIF-1α [339]. Increased stabilization of HIF-2α in severely hypoxic astrocytes could therefore be due to increased Siah2-dependent degradation of PHD1 and PHD3. Finally, as mentioned above, HIF-1α is preferentially degraded as a result of its interaction with HAF, and Hsp70 and CHIP [103, 104]. The higher expression of HIF-2α compared to HIF-1α in astrocytes exposed to severe compared to intermediate hypoxia could be due to higher expression of these proteins in astrocytes exposed to severe hypoxia, which would lead to increased degradation of HIF-1α. However, whether these proteins (Siah2, the PHDs, HAF, Hsp70, and CHIP) are differentially expressed in astrocytes exposed to intermediate or severe hypoxia was not tested in our study.

The higher stabilization of HIF-2α could be accompanied by higher transcriptional activity. FIH can inhibit the HIFs at lower oxygen tensions compared to the PHDs and has a higher sensitivity for HIF-1α [100, 340]. FIH regulates the interaction between HIFα subunits and their transcriptional coactivator CBP/p300 [98], so active FIH at the very low oxygen tensions of severe hypoxia would lead to a decrease of HIF-1α but not HIF-2α transcriptional activity. This could indicate that HIF-2α is not just more abundant but also more transcriptionally active in severely hypoxic astrocytes.

Whether this preferential stabilization and potentially higher transcriptional activity of HIF-2α is translated to differential gene expression in astrocytes exposed to severe compared to intermediate hypoxia is unknown. However, some studies suggest that this might be the case. It has been shown that the HIFα transactivation domains are important for regulating target gene specificity [341]. More specifically, the NAD was shown to be important for the regulation of specific HIF-1α and HIF-2α target genes, while the CAD was important for the transcription of common target genes [341]. A study aimed at investigating whether the HIFs regulate different sets of genes in renal cell carcinoma found that both HIF-1α and HIF-2α regulate several classical hypoxia-inducible genes, but that glycolytic genes are regulated exclusively by HIF-1α [342]. Interestingly, even though one would expect both HIFs to be important for adaptations at environmental hypoxia, Tibetan populations living in high altitudes with low oxygen availability have accumulated mutations specifically on HIF-2α and not HIF-1α [343-345]. Finally, in renal cell carcinoma, HIF-1α and HIF-2α have opposing roles in regard to tumor growth, with HIF-1α being anti-tumorigenic and HIF-2α being pro-tumorigenic [121, 122]. Despite these reports, it is still unclear whether HIF-1α and HIF-2α have unique target genes.

Apart from evaluating the stabilization of HIFs and the expression of HIF-regulated genes, we also wanted to study other biological responses of astrocytes as a result to growth in low oxygen conditions. Because it is known that astrocytes produce and secrete several cytokines in response to various stimuli, we evaluated the

production of a panel of cytokines in hypoxic astrocytes. We found that astrocytes exposed to intermediate hypoxia expressed TGF-β and IL-3. Astrocyte-derived TGF-β has been shown to increase glioma cell invasion *in vitro* [346], while exogenous TGF-β increased the stem-like phenotype of glioma cells [347]. We also found that astrocytes exposed to severe hypoxia produced VEGF-A, angiogenin, and IL-1α. Exogenous VEGF has been shown to induce proliferation and migration of glioma cells, as well as an upregulation of CXCL12 and CXCR4 expression [348]. Treatment with exogenous CXCL12 was shown to increase the proliferation of glioma cells *in vitro* [166]. Angiogenin has been shown to increase the proliferation of glioma cells when added in the cell culture medium [349]. Both angiogenin and IL-1α were shown to be upregulated in glioma cells in response to bevacizumab treatment [350]. This suggests that astrocytes might also upregulate these cytokines in response to treatment and not just in response to hypoxia. Finally, treatment of glioma cells with IL-1α has been shown to alter their secretome and change the expression of proteins related to cell survival, invasion, and angiogenesis [351], suggesting that it has various ways of increasing the tumorigenic properties of glioma cells. All in all, cytokines that we identified in our study have been implicated in glioblastoma biology and could be responsible for the changes we observed when glioma cells were cultured on matrix produced by hypoxic astrocytes.

In paper I we showed that matrix from irradiated astrocytes promotes glioma cell stemness. In paper II, we addressed again the effect of ECM on glioma cell properties. We found that matrix from astrocytes exposed to intermediate hypoxia increased the proliferation of glioma cells while matrix from severely hypoxic astrocytes increased the drug efflux capacity of glioma cells, one measure of cell stemness [66, 328]. It would be interesting to explore further if different levels of hypoxia induce different phenotypes in glioma cells cultured in the presence of matrix or medium from hypoxic astrocytes, further supporting that glioma cells are highly plastic. Conversely, the different response of astrocytes to hypoxia could also be indicating that they acquire a spectrum of phenotypes in the presence of various oxygen tensions and that astrocyte reactivity has several aspects. In either case, if future studies support our observation that hypoxic astrocytes support glioma cell proliferation and stemness then targeting this cross-talk would provide a novel therapeutic opportunity.

Here, we examined how matrix from hypoxic astrocytes affects properties of glioma cells cultured in normoxia. It would be interesting to study if hypoxic astrocytes affect the cellular adaptation of glioma cells to hypoxia. In Paper I, we showed that treatment of glioma cells with TGM2, a protein that was secreted in the ECM by irradiated and reactive astrocytes, led to increased signaling through the integrin-β1 pathway. The integrin pathway has also been implicated in hypoxia regulation in glioblastoma [352]. Whether hypoxic astrocytes produce TGM2 as part of their reactive phenotype was not addressed in this study. However, it would be interesting

to evaluate the production of TGM2 by astrocytes in hypoxia and study the integrin-β1 pathway in relation to the effect of the hypoxic astrocyte-derived matrix on glioma cell properties.

In this study, we examined the effect of temozolomide treatment on astrocyte reactivity. Interestingly, we found that astrocytes become reactive in response to temozolomide treatment, in a similar manner as irradiated astrocytes in papers I and II and hypoxic astrocytes in paper II. A few studies have implicated astrocytes in glioma cell resistance to temozolomide [172, 353]. These observations together suggest the astrocytes might have a tumor-protective role in response to chemotherapy treatment. Studying this cross-talk could provide new opportunities for therapeutically targeting the treatment resistant glioma cells.

In conclusion, we showed that astrocytes become reactive in response to hypoxia, a common feature of glioblastomas. Glioma cells cultured on the matrix produced by hypoxic astrocytes showed an increase in proliferation and drug-efflux capacity, two pro-tumorigenic properties. More studies are needed to further elucidate the cross-talk between hypoxic astrocytes and glioma cells in order to develop new strategies for the treatment of glioblastoma.

Conclusions from Papers I and II

In papers I and II we have established the importance of one component of the glioma microenvironment, namely the astrocytes, in glioblastoma progression. We have shown that both intrinsic factors of the glioblastoma microenvironment such as hypoxia, as well as extrinsic factors such as radiation or chemotherapy treatment, render tumor-associated astrocytes reactive. Reactive astrocytes then produce ECM that affects glioma cell properties such as proliferation, drug-efflux, self-renewal, and radioresistance. In paper I we have identified at least one protein secreted by irradiated astrocytes to be involved in this process, namely TGM2, and in paper II we have proposed various cytokines produced by hypoxic astrocytes as potential drivers of glioma cell tumorigenicity.

Both papers focus on the astrocytes' response to insult: in paper I, radiation, and in paper II, hypoxia and to a lesser extent temozolomide treatment. We found that all treatments induced somatic hypertrophy and upregulation of vimentin expression for up to 72 hours in primary human astrocytes treated *in vitro* with radiation, hypoxia or temozolomide. Interestingly, no morphological changes were observed in astrocytes 4 hours after irradiation or temozolomide treatment (unpublished data), suggesting that more time is needed for the cells to acquire these phenotypes. Both irradiation and temozolomide treatment induced somatic hypertrophy and upregulation of vimentin within the same time frame, for up to 72 hours after treatment. On the contrary, intermediate and severe hypoxia led to an increase in

vimentin expression after 24 hours which was then replaced by an increase in cell surface. Similar to the morphological changes, both radiation treatment and hypoxia led to an increase in the production of cytokines from reactive astrocytes, although different cytokines were produced in response to irradiation, and intermediate and severe hypoxia.

This variability in the induction of reactivity features might arise from the activation of different pathways in response to the various injuries. Temozolomide treatment seems to render astrocytes reactive in a similar manner as irradiation, albeit to a lesser degree. This is not surprising as both irradiation and temozolomide treatment induce DNA damage that leads to the generation of DSBs and the recruitment of ATM [354, 355]. Interestingly, a study on the additive effect of temozolomide to radiation treatment, found that irradiation led to G2/M arrest in glioma cells faster than temozolomide treatment did [356]. This suggests that the difference we observed in the features of reactive astrogliosis between irradiated and temozolomide treated astrocytes in paper II might be reflecting a delay in the response of the cells to the stimulus and not a difference in the degree of the response. Hypoxia on the other hand seems to also induce astrocyte reactivity but in a slightly different manner compared to the other two insults. For instance, matrix from severely hypoxic astrocytes, similar to matrix from irradiated astrocytes, affected the drug efflux of glioma cells cultured on it. However, matrix from astrocytes exposed to intermediate hypoxia increased the number and size of colonies that glioma cells form when cultured on it, a phenotype that we did not observe when cells were cultured on matrix from irradiated astrocytes. These observations might be explained by the production of different proteins from differentially treated astrocytes that when incorporated in the matrix affect glioma cell properties in different ways.

The variability in the reactivity responses that astrocytes exhibit could therefore be a consequence of their phenotypic plasticity. Similar to the heterogeneity and plasticity that characterizes glioma cells and GSCs [37-39], astrocytes might also exhibit heterogeneity and plasticity between phenotypes. Notably, a binary characterization of reactive astrocytes as neurotoxic or neuroprotective has been suggested [357, 358]. However, this strict categorization disregards a vast spectrum of phenotypes that stromal cells might adopt and hinders the efforts to fully understand their response to insults and implications to tumor biology. As a result, binary polarization states such as A1-A2 astrocytes or M1-M2 microglia are gradually being replaced by theories that incorporate more plasticity even within the stromal compartment of the tumor microenvironment [159, 359]. This plasticity in stromal phenotypes has been documented for macrophages in the context of glioblastoma, in a study that identified that the irradiated brain microenvironment shifts tumor-associated macrophages towards a tumor-supportive phenotype [143]. The role of the microenvironment is also highlighted by the discovery that the

microenvironment of the various segments of the gut leads to an oncogenic or tumor-suppressive effect of mutant p53 [360].

Upregulation of TGM2 and of cytokines is not the only response of reactive astrocytes. Our analysis of the proteome of irradiated astrocytes revealed other proteins that might be interesting to study in the context of glioma cell stemness maintenance. Interestingly, reactive astrocytes have been shown to also produce higher levels of CD44 variant isoforms, such as CD44v6 [361]. This isoform of CD44 has been successfully targeted by the use of specific antibodies or peptides that block signal transduction from it [239, 362, 363]. Blocking the crosstalk between reactive astrocytes and cancer cells by inhibiting TGM2, and cytokine or other signaling, such as signaling by CD44v6, is one way of targeting reactive astrocytes. Alternatively, the focus could lie in efforts to revert the reactive phenotype of astrocytes. This approach would revert astrocytes to an anti-tumorigenic phenotype, it would diminish the effect of treatments or hypoxia on these cells, and would increase the effect of treatments.

Papers I and II mostly focused on the effects that reactive astrocyte-derived ECM has on glioma cells. However, astrocytes might be affecting glioma cells either by direct cell-cell contact, as it has been shown in other studies [172], or by soluble factors secreted in the microenvironment. Interestingly, in another study from our lab, we showed that irradiated and hypoxic astrocytes secrete the soluble delta-like noncanonical Notch ligand 1 (DLK1) [364]. Treatment of glioma cells with recombinant DLK1 increased proliferation and stemness, including self-renewal, colony formation, and stem cell marker expression, possibly by stabilizing HIF-2α expression. Overexpression of DLK1 led to a decrease in survival in a mouse model of glioma [364]. Astrocytes could therefore be affecting glioma cell properties by altering the ECM and their secretome, or by direct cell-cell interactions with glioma cells.

All in all, our studies on astrocytes reveal a role of this cell type in glioblastoma therapy resistance and recurrence. Our data highlight the importance of further studies into the response of this and other stromal cell types to irradiation, chemotherapy treatment, and hypoxia in search of new therapeutic opportunities for glioblastoma patients.

Paper III: CD44 interacts with HIF-2α to modulate the hypoxic phenotype of perinecrotic and perivascular glioma cells

In this study, we aimed to investigate the role of CD44 signaling in hypoxic and pseudo-hypoxic regions in glioblastoma. We showed that CD44 cleavage by

ADAM17 was enhanced by hypoxia and led to the release of the CD44ICD which interacted directly with HIF-2α, but not HIF-1α. Blocking the cleavage of CD44 led to a decrease in stemness features of glioma cells in hypoxia and an increase in the differentiation of primary glioma cells. Interestingly, CD44 was expressed both in the well-oxygenated perivascular niche as well as in the poorly oxygenated perinecrotic niche *in vivo*, along with HIF-2α alone or with both HIF-1α and HIF-2α, respectively. Moreover, the expression of the intracellular fragment CD44ICD led to increased stabilization and transcriptional activity of HIF-2α in the presence of 1% and 5% oxygen. All in all, we showed that CD44 signaling is implicated in maintaining the GSC phenotype by stabilizing HIF-2α and is active in the perivascular and the perinecrotic niche.

Discussion

The relationship between hypoxia and stemness in glioblastoma has been extensively studied. A link between the two is well-established, with hypoxia being a known regulator of glioma cell stemness [205-211]. Similarly, the perivascular niche and oxygen tensions higher than 1% have also been shown to increase glioma cell stemness [207, 212-215]. The reported stabilization of the HIF factors in oxygen tensions higher than 1% is referred to as pseudo-hypoxia. In this study, we addressed how stemness phenotypes are differentially regulated in different niches, namely the perivascular and the perinecrotic niche, and whether this regulation is dependent on CD44 signaling. CD44 has been used as a stem cell marker in glioblastoma studies [246]. In a previous study, CD44 was shown to be expressed in the perivascular niche *in vivo* and the CD44ICD stabilized HIF-2α protein expression by interacting with the transcriptional co-activator CBP/p300 *in vitro* in glioblastoma [175]. With this study we aimed to further investigate the interaction of CD44ICD with HIF-2α in glioma and its role in the perivascular and perinecrotic niche.

The initial cleavage of CD44 has been previously shown to be mediated by ADAM10 and ADAM17 [225, 226]. Here we showed that hypoxia leads to the generation of mature ADAM17, but not of mature ADAM10, which then leads to increased CD44 cleavage and the generation of the CD44ICD. The CD44ICD was then found to interact with HIF-2α, but not HIF-1α, to stabilize HIF-2α protein expression both in hypoxic and pseudo-hypoxic conditions and to be at least partially responsible for increased stemness phenotypes of glioma cells in hypoxia. These findings suggest that in the perinecrotic niche, HIF-2α is stabilized, due to the lack of oxygen, leading to an increase in ADAM17 activity. This leads to increased CD44ICD generation which, in turn, leads to increased stabilization of HIF-2α and intensification of hypoxia related phenotypes, such as stemness. In the perivascular niche, the generation of the CD44ICD leads to the stabilization of HIF-2α and the potentiation of its transcriptional activity. As a result, CD44ICD-

expressing cells acquire a pseudo-hypoxic phenotype even in the presence of oxygen in the perivascular niche.

The mechanism of ADAM17 stabilization by hypoxia and pseudo-hypoxia is not fully understood yet. In human lung fibroblasts, ADAM17 transcription was regulated by the hypoxia-induced binding of C/EBPβ to the gene's promoter [365]. In rheumatoid arthritis, ADAM17 mRNA levels were increased under hypoxia by direct binding of HIF-1α in the promoter region [366]. However, the authors did not examine the effect of HIF-2α in ADAM17 regulation. An increase in ADAM17 expression was regulated by HIF-1α, but not by HIF-2α, in response to 1% oxygen and by endoplasmic reticulum stress in response to severe hypoxia (<0.1% oxygen) in human glioblastoma, breast and colon cancer cells, fibroblasts, and kidney cells [367]. Here, we showed that ADAM17 expression is at least partially regulated by HIF-2α in hypoxia. However, the mechanism of this regulation is unknown. If ADAM17 activity is increased by pseudo-hypoxia has yet to be examined. We detected the HIF-2α protein in glioma cells cultured at 5% oxygen, and this could suggest that HIF-2α might regulate ADAM17 levels also at this oxygen tension.

In this study, we addressed the reason for the increased protein stabilization of HIF-2α in response to ADAM-mediated cleavage. By using the ADAM inhibitor TAPI-2 in the presence of 2,2'-dipyridyl (DIP), a prolyl hydroxylase inhibitor, and MG132, a proteasome inhibitor, we showed that the stabilization of HIF-2α following an ADAM-mediated cleavage of CD44 is dependent on hydroxylation by the PHDs and on escaping proteasomal degradation. Whether other ADAM substrates or indirect effects of the CD44ICD generation are involved in the process of stabilization of HIF-2α protein expression is still not fully understood.

Interestingly, we also found that CD44ICD interacts with and stabilizes HIF-2α but not HIF-1α. In previous studies it was shown that CD44ICD increases the activity of the transcriptional coactivator CBP/p300 [227] and by doing so it enhances HIF-2α transcriptional activity in glioblastoma [175]. In accordance with these observations, here we show that the preferential stabilization of HIF-2α and not HIF-1α by CD44ICD is at least partially dependent on the FIH-mediated hydroxylation of HIF-1α, a process that leads to abrogation of the interaction between HIF-1α and CBP/p300 [98]. These data explain previous findings on stabilization of HIF-2α in the perivascular and not just the perinecrotic niche, as well as findings on stabilization of HIF-2α preferentially in cancer stem cells since they express CD44 [175, 207].

Apart from being a stem cell marker, CD44 also characterizes astrocytes [368-370]. Moreover, it has been shown that reactive astrocytes express higher levels of CD44 variant isoforms compared to normal astrocytes [361]. In our mouse model of glioma, CD44 was used as a stemness marker, marking cancer cells in the perivascular and perinecrotic niche and being generally absent in the bulk of the tumor, similar to previous reports about CD44 expression in this mouse model

[175]. However, in Paper II we showed that astrocytes are also present in hypoxic areas in our mouse model of glioma**.** Moreover, astrocytes are surrounding blood vessels and are therefore present in the perivascular niche [153]. To confirm that the CD44 positive cells in the tissue samples of this study were in fact tumor cells and not astrocytes, we could have co-stained with GFAP, to stain for astrocytes, and with PDGF-B, since the tumor cells in our model are overexpressing it, or with Olig2, since the majority of the tumor cells in our model are Olig2 positive [327].

Identifying other binding partners of CD44ICD apart from HIF-2α could further elucidate how it is involved in maintaining glioma cell stemness. It is known that CD44ICD interacts with CBP/p300 [227], as well as with the Ezrin/radixin/moesin family of proteins [371, 372]. In our lab we are currently working on detecting other binding partners of CD44ICD and characterizing their role in glioblastoma biology. By doing so, we aim to identify pathways that are active primarily on glioma cells or GSCs and therefore propose therapeutic targets that will have higher specificity for tumor cells.

In conclusion, in this study we showed that CD44 is present in both hypoxic and perivascular areas in glioblastoma and that CD44 cleavage is induced by ADAM17 in hypoxia, leading to the stabilization of HIF-2α. Importantly, the expression of the CD44ICD induced hypoxic signaling at the physiological oxygen tensions of the perivascular niche. These data suggest that expression of CD44 by GSCs in the perivascular and the perinecrotic niche activates the hypoxic signaling pathway in these cells and mediates their stem-like phenotypes. All in all, we propose CD44 as a promising therapeutic target for glioblastoma.

Paper IV: The p75 neurotrophin receptor enhances HIF-dependent signaling in glioma

In this study we aimed to investigate the role of $p75^{NTR}$ signaling in hypoxia in glioblastoma. We found that $p75^{NTR}$ is expressed in the perinecrotic niche *in vivo* and it is upregulated by hypoxia in glioma lines. Using an siRNA to downregulate the expression of $p75^{NTR}$ or a specific $p75^{NTR}$ inhibitor, we showed that inhibition of $p75^{NTR}$ decreases the stabilization of HIF-1α and HIF-2α, as well as the expression of several HIF target genes. Overexpression of $p75^{NTR}$ led to an increase in HIF-1α and HIF-2α stabilization as well as to increased HIF transcriptional activity. Moreover, we showed that downregulation of $p75^{NTR}$ mRNA expression or pharmacological inhibition of $p75^{NTR}$ signaling decreased the drug-efflux capacity, the stem-cell marker expression and the size of spheres generated by glioma cells in hypoxia. Downregulation of $p75^{NTR}$ also negatively affected migration of glioma cells in hypoxia. All in all, we showed that $p75^{NTR}$ signaling is upregulated in response to hypoxia, it regulates the stabilization and transcriptional activity of the

HIFs and is associated with hypoxia-induced glioma phenotypes such as stemness and migration.

Discussion

Signal transduction through the $p75^{NTR}$ receptor follows a similar sequence of cleavages as CD44. Ligand binding leads to an ADAM-mediated cleavage of the extracellular domain and the generation of the membrane bound CTF, which is followed by a γ-secretase-mediated cleavage and the generation of the p75ICD [257-260]. In paper III we elucidated the prominent role of CD44 in maintaining stemness through the generation of the CD44ICD under hypoxic and pseudo-hypoxic conditions. Here, based on the similarities between the CD44 and $p75^{NTR}$ cleavage cascades we aimed to identify the role of $p75^{NTR}$ in hypoxia-related phenotypes in glioblastoma.

Upregulation of $p75^{NTR}$ in response to hypoxia has been studied in the context of ischemia. $P75^{NTR}$ has been reported to be upregulated in response to hypoxia in murine endothelial cells *in vivo* [373], in human retinal pigment epithelial cells *in vitro* [374], and in mouse neurons *in vivo* [375]. In contrast, very few studies have addressed the interplay between $p75^{NTR}$ and hypoxia in the context of glioma. In this study, we found that $p75^{NTR}$ is highly upregulated in response to hypoxia *in vitro* and it is specifically expressed in perinecrotic and thus hypoxic areas in a mouse model of glioma *in vivo*. How $p75^{NTR}$ expression is induced by hypoxia is not known. One possibility would be that hypoxia induces an increase in *NGFR* transcription, the gene encoding for $p75^{NTR}$; however, we found no evidence of that in our cell systems (unpublished data). In fact, knocking down the HIFs did not affect the $p75^{NTR}$ protein levels in hypoxia, suggesting that the upregulation of $p75^{NTR}$ by hypoxia is not HIF-dependent. Another possibility is that $p75^{NTR}$ is protected from degradation under hypoxia, perhaps by interacting with co-receptors such as TrkA. Interestingly, interaction between TrkA and $p75^{NTR}$ creates high affinity binding sites for NGF [256], which is secreted by astrocytes in response to hypoxia [376]. However, whether this binding stabilizes the receptor is unknown.

While $p75^{NTR}$ expression is not regulated by the HIFs, we found that $p75^{NTR}$ increases HIF-1α and HIF-2α stabilization and transcriptional activity in glioma cells *in vitro*. These findings are in line with a previous study that reports increased stabilization and transcriptional activity of HIF-1α in hypoxia by $p75^{NTR}$ overexpression in fibroblasts and neurons [261]. In that study, ADAM- and γ-secretase-mediated cleavage of $p75^{NTR}$ in hypoxia was shown to stabilize the levels of the ubiquitin ligase Siah2, which is responsible for the degradation of the PHDs, and as a result to stabilize HIF-1α protein expression levels. The effects of $p75^{NTR}$ on HIF-2α stabilization were not addressed in that study. In this study, we found that the mRNA levels of *HIF-1A* and *EPAS1*, the gene encoding for HIF-2α, were not affected by siRNA-mediated knockdown of $p75^{NTR}$ (unpublished data). This

indicates that $p75^{NTR}$ induces protein stabilization rather than increased HIFα gene transcription. Consequently, $p75^{NTR}$ could be inhibiting HIFα proteasomal degradation by inducing PHD degradation in glioma cells too. Another possibility, is that $p75^{NTR}$ mediates HIF stabilization by leading to Akt activation. Phosphorylation and activation of Akt by $p75^{NTR}$ has been previously reported [377], while regulation of HIF-1α stabilization by Akt is also documented [107, 108]. It cannot be excluded that $p75^{NTR}$ also affects the FIH/HIFα interaction, although since $p75^{NTR}$ regulates both HIF-1α and HIF-2α in a similar manner, the involvement of FIH seems less likely.

In glioblastoma, the $p75^{NTR}$ receptor is a well-established mediator of migration and invasion [274-277]. This study further supports the role of $p75^{NTR}$ in glioma cell migration, especially in hypoxia. Moreover, we provide evidence that $p75^{NTR}$ signaling is necessary for the maintenance of glioma cell stemness, as glioma cells treated with siRNA targeting $p75^{NTR}$ or a $p75^{NTR}$ inhibitor show reduced drug efflux, stem cell marker expression, and sphere area in hypoxia. Identifying downstream effectors of the $p75^{NTR}$-mediated migration and stemness could assist in the development of targeted therapies for glioma cells with these aggressive phenotypes.

In conclusion, in this study we showed that $p75^{NTR}$ is expressed primarily in the perinecrotic niche of murine gliomas, its expression is upregulated by hypoxia *in vitro*, and it regulates HIFα stabilization and transcriptional activity. Moreover, increased $p75^{NTR}$ signaling by hypoxic glioma cells leads to increased migration and stemness. All in all, targeting $p75^{NTR}$ would minimize several hypoxia-related phenotypes that are associated with glioblastoma aggressiveness.

Conclusions from Papers III and IV

In papers III and IV we studied the signaling involved in the maintenance of the hypoxic and pseudo-hypoxic glioma cells, with a focus on the maintenance of aggressive phenotypes such as stemness and migration.

In these studies, we showed that CD44 and $p75^{NTR}$ signaling is important in the maintenance of hypoxic phenotypes by glioma cells in the perivascular and perinecrotic niche. In our lab we have identified that cleavage of DLK1, another substrate of ADAM17 [378], also generates an intracellular fragment in a HIF- and ADAM-dependent manner in response to hypoxia [379]. Once generated, the DLK1-ICD translocates to the nucleus of glioma cells and mediates cellular adaptations to hypoxia such as colony formation, stem cell marker expression, increased glucose consumption, and invasion [379]. Overexpression of a cleavable form of DLK1 led to more invasive tumor growth in a mouse model of glioma [379].

Importantly, DLK1 was found expressed in the nucleus of cells in the perivascular and the perinecrotic niche of murine gliomas [379].

Based on these studies, we propose that targeting the cleavage of these transmembrane proteins (DLK1, CD44, and $p75^{NTR}$) wound inhibit the signaling pathways that drive hypoxia-related phenotypes. This would in turn address features of glioblastoma such as infiltrative growth, therapy resistance, and tumor recurrence and consequently improve patient outcomes.

One way of targeting these proteins is to block the ligand-receptor interaction. In glioblastoma, CD44 interacts with osteopontin, and upon ligand binding increases stemness features *in vitro* and leads to more aggressive tumors *in vivo*, in a CD44ICD-dependent manner [175]. Recently, verbascoside, an inhibitor that impedes CD44 dimerization, was identified [380]. The inhibitor decreased ligand binding and subsequent CD44ICD generation. The inhibitor was shown to reduce proliferation and stemness features of glioma cells, such as colony formation, stem cell marker expression, and drug efflux, and led to prolonged survival in an orthotopic mouse model of glioma [380]. In paper IV we used the $p75^{NTR}$ inhibitor Ro 08-2750 to reduce signal transduction from the receptor. Ro 08-2750 blocks the interaction between $p75^{NTR}$ and NGF by binding to NGF and inducing a conformational change that no longer allows NGF to bind to $p75^{NTR}$ [381]. The successful preclinical studies using these inhibitors might encourage the design of molecules that cross the BBB and target the receptor-ligand binding of CD44 and $p75^{NTR}$ in glioblastoma.

Another way of targeting CD44 and $p75^{NTR}$ signaling is to block the initial cleavage of the receptors by MMPs or ADAMs. MT1-MMP is one of the metalloproteases that cleaves CD44 [224]. Cyclosporin A was shown to affect glioma cell invasion by affecting MT1-MMP translocation to invadopodia in glioma cells [382], but whether this affects CD44 cleavage is not known. Conversely, inhibition of ADAMs has been used more broadly to block substrate cleavage. In paper III we use TAPI-2, TMI-1, and GI 254023X to block the initial cleavage of CD44 by ADAMs. TAPI-2 inhibits both ADAM10 and ADAM17 [383], GI 254023X is more specific for ADAM10 than ADAM17 [384], and TMI-1 inhibits ADAM17 amongst other MMPs [385]. These inhibitors also affect the proteolytic cleavage and signal transduction though Notch, so data generated using them should address the effect of Notch inhibition in the assesed phenotypes. For instance, in paper III we evaluated the effect of the Notch ICD in inducing the HIF-mediated transcriptional activity. Overall, several different approaches have been used to target the ADAMs, including small molecule inhibitors and antibodies; however, they have not proven effective in clinical trials [386, 387].

In paper III we used the γ-secretase inhibitor DAPT to block the generation of the CD44ICD. Using γ-secretase inhibitors to treat glioblastoma is not a new concept, as discussed in a previous chapter. Several clinical trials have used γ-secretase

inhibitors to target Notch signaling in glioblastoma and other cancers. [388-391]. The Notch pathway is involved in stemness maintenance and tumor growth and, like the CD44 and $p75^{NTR}$ pathways, also leads to the generation of an intracellular domain in an ADAM- and γ-secretase-dependent manner [392, 393]. Therefore, clinical trials that target the generation of the Notch ICD, also target the generation of CD44ICD and p75ICD. Phase 0 and I trials with the γ-secretase inhibitor RO4929097 in combination with bevacizumab or temozolomide and radiotherapy showed that the inhibitor was well tolerated by glioblastoma patients, it crossed the BBB, and blocked Notch activation [394, 395]. However, a subsequent phase II trial of the inhibitor in recurrent glioblastoma showed minimal improvement in patient outcomes [302]. Moreover, long-term treatment with γ-secretase inhibitors has severe adverse effects and treatment regiments implementing γ-secretase inhibitors should take this into consideration. Although γ-secretase has proven difficult to target successfully in clinical trials, the biological importance of γ-secretase-mediated cleavages renders γ-secretase inhibitors attractive drug candidates for glioblastoma.

Glioblastomas are characterized by high intratumoral heterogeneity [37]. In fact, hypoxia was one of the gene signatures that was heterogeneously expressed within each tumor of a small patient cohort [37]. Moreover, cells from one patient were shown to exhibit a spectrum of stemness-associated genes, suggesting that microenvironmental factors might be affecting the GSC phenotype [37]. More recent studies have shown that glioblastoma cellular states, including stemness, are highly plastic, and this plasticity is partially dictated by microenvironmental cues, including by hypoxia [38, 39]. In paper III we showed that GSC of the perivascular and perinecrotic niche might differentially stabilize the HIFs in a CD44ICD-dependent manner. Similarly, we showed nuclear localization of DLK1 in the perivascular and perinecrotic niche, and even colocalization with CD44 in both niches [379]. In paper IV we reported $p75^{NTR}$ to be associated with stemness phenotypes in the perinecrotic niche, but to be absent from the perivascular niche. These observations suggest that the two spatially distinct GSC populations, the one located in the perivascular and the other in the perinecrotic niche, might represent different manifestations of the same phenotypic state. These manifestations are dictated by different microenvironmental cues, such as variations in oxygen tension. Interestingly, the same signaling pathways can be differentially regulating properties of glioma cells residing in specific niches, such as therapy resistance, as is the case with Notch1 signaling in the perivascular niche versus the tumor microtube-dependent multicellular networks [396]. Therefore, targeting all niches will be crucial for the successful elimination of all aggressive phenotypes.

Further studies on these proteins will provide more details on their role in maintaining glioma cell stemness under different microenvironmental conditions and will motivate the generation of targeted therapies for glioblastoma patients.

Overall conclusions and future perspectives

In the clinic, glioblastoma is treated aggressively with surgery, chemotherapy, radiotherapy, and tumor-treating fields. The infiltrative nature of these tumor, the therapy resistance, and the frequent incidence of recurrences present unsurmountable hurdles in the treatment of glioblastoma patients, most of who succumb from the disease in a short period of time. There is therefore a dire need for new approaches in the treatment of this incurable disease.

Tumors are complex systems where cancer cells and cells of the microenvironment closely interact and affect each other's properties. This close-knit microenvironment is highly affected by therapeutic interventions during the treatment of the primary tumor, but also by intrinsic characteristics of the microenvironment itself. This is evident in studies of the clonal evolution of glioblastoma and other tumors during therapy, where regardless of the tumor type, treated or recurrent tumors show different evolutionary trajectories compared to the ones predicted from the primary tumor [41-43, 397]. We need to fully understand the treatment- and microenvironment-induced heterogeneity, to provide novel treatment options to glioblastoma patients. The work included in this thesis directly addresses the need for a better understanding of the mechanisms that contribute to glioma heterogeneity in response to treatments, such as radiation or temozolomide, and microenvironmental factors, such as hypoxia.

In papers I and II we delineate the pro-tumorigenic effects of radiation therapy and hypoxia on tumor-associated astrocytes. These studies implicate irradiated and hypoxic, and consequently reactive, astrocytes in glioblastoma stemness and tumor aggressiveness. It will be imperative to understand all the potential pathways involved in the cross-talk between reactive astrocytes and glioma cells. To this end, further studying the ECM composition as well as the secretome of treated and hypoxic astrocytes will better outline the role of this cell type in glioblastoma.

Another interesting question that remains to be addressed is whether differentially treated astrocytes maintain different aspects of glioma stemness. If that is the case, then several approaches need to be developed in order to perturb astrocyte reactivity and the reciprocal signaling between reactive astrocytes and glioma cells. In paper I, we propose that targeting the astrocyte-derived TGM2 could inhibit the effects of reactive astrocytes on glioma cell stemness. Another approach to target reactive astrocytes would be to revert their reactive phenotype. Reducing or eliminating treatment- or hypoxia- induced astrocyte reactivity could reduce cancer stemness and improve treatment outcomes.

In paper II, we studied the effect of temozolomide on astrocyte reactivity. Considering the vast use of this chemotherapeutic in patient care, further studies on the response of astrocytes to this and other chemotherapeutic drugs might lead to

the development of therapies that when administered concomitantly could improve patient outcomes.

Finally, even though the focus of papers I and II was the astrocytes, several other stromal cells exist in the glioma microenvironment. A recent study showed that macrophage-derived oncostatin M shifts glioma cells towards the more aggressive mesenchymal-like state [38], and tumor cells of this state led to reciprocal changes in the macrophages [398]. Our studies show that targeting astrocyte reactivity and therefore the cross-talk between the treated or hypoxic astrocytes and glioma cells can disturb the maintenance of the GSC population. This approach could lead to better responses to therapies and to a decrease in the risk for recurrence. These and other studies underline that understanding the cross-talk between stromal and glioma cells is a necessary step for successfully treating glioblastomas.

Hypoxia is a hallmark of glioblastoma that not only affects stromal cells, but it also greatly influences glioma cells. Notably, the HIFs, the transcription factors that regulate the cellular responses to hypoxia, have been implicated in the maintenance of GSCs. In papers III and IV we provide further evidence in support of the role of hypoxia in glioma cell stemness, by signaling mediated by CD44 and $p75^{NTR}$. Our studies show that targeting ADAM- and γ-secretase-dependent cleavage of these transmembrane proteins could inhibit glioma cell stemness and migration. For this approach to succeed, other targets of these enzymes should be studied in relation to these glioma phenotypes. Moreover, further studies will provide more evidence on whether ADAM and γ-secretase inhibition are viable targets or if the focus should be on targeting downstream molecules that are more specific for the CD44 or $p75^{NTR}$ pathways.

In paper III we revealed that the differential stabilization of the HIFs in hypoxic and pseudo-hypoxic environments is regulated by the generation of the CD44ICD. While we addressed how CD44ICD generation is induced by hypoxia, its induction by pseudo-hypoxia remains elusive. Clarifying this could further improve our understanding of the activation of the CD44 signaling pathway in the various GSC niches. Although we described that CD44ICD is implicated in glioma cell stemness by interacting with HIF-2α, further studies will show if interactions with other binding partners also affect stemness or other aspects of glioblastoma biology. Another important question that should be addressed is whether spatially distinct GSC populations are also functionally different. If that is the case, then other microenvironmental factors, apart from the differential stabilization of HIF-1α and HIF-2α, present in each niche could be responsible for this plasticity.

Collectively, the work included in this thesis addresses the effects that extrinsic factors, such as irradiation and temozolomide treatment, or intrinsic factors of the microenvironment, such as hypoxia, have on stromal cells and tumor cells in glioblastoma. Our studies indicate that both stromal cells and glioma cells are highly plastic, and they exhibit a spectrum of phenotypes in response to different

microenvironmental cues (Figure 7). It is essential to understand this plasticity and the interactions between the various phenotypic states of stromal and glioma cells. Only by doing so will it be possible to specifically target the phenotypes of glioma cells that render treatment impossible, namely the increased infiltrative growth and increased stemness.

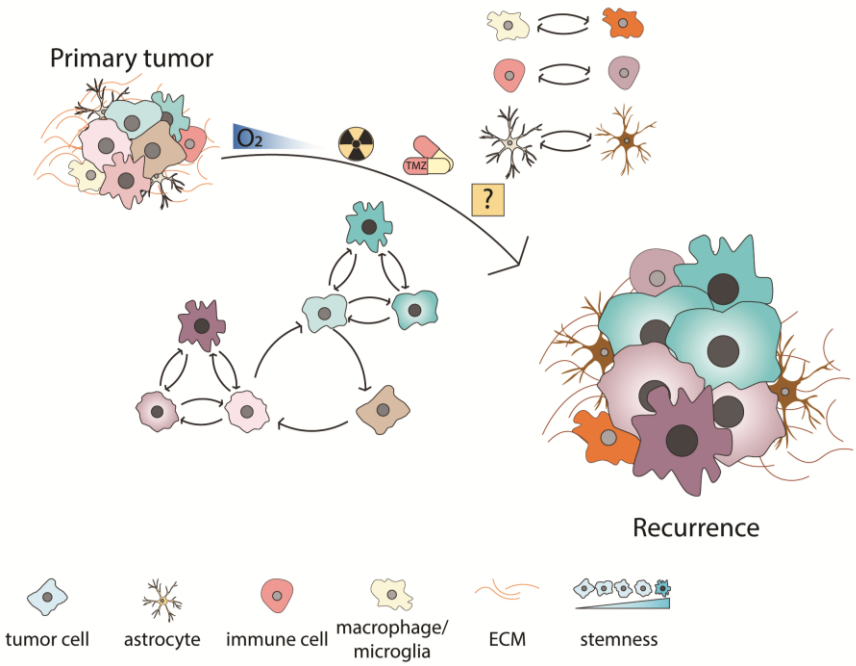

Figure 7 Tumor and stromal cell plasticity in response to therapy and treatment in glioblastoma
Our studies support a model where the increasing hypoxia generated in the primary tumor, treatment of the primary tumor with radiation or temozolomide, or other yet unknown microenvironmental cues lead to increased plasticity of glioma cells and stromal cells. As a result, glioblastomas are repopulated by cells with more aggressive properties and treatment-resistant recurrent tumors arise.

The complexity that characterizes tumor lesions, with dynamic changes occurring in the glioma and stromal cell compartment, dictate the need for a holistic approach in the treatment of glioblastomas. This concept leads to the abandonment of the traditional monotherapy approach and instead emphasizes the need for targeted therapies that concomitantly will lead to improved patient outcomes. This thesis contributes to a growing list of potential therapeutic targets for glioblastoma and provides a framework for future studies that will further elucidate the role of the treated and hypoxic microenvironment in maintaining various glioma cell phenotypes.

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