MODULATION OF SYSTEM X_C- MEDIATED GLUTAMATE RELEASE IN GLIOBLASTOMA MULTIFORME VIA THE EXTRACELLULAR MATRIX: THE AGONY AND THE xCTASY

By

JOELLE MARTIN

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HARALD SONTHEIMER, COMMITTEE CHAIR

MICHELLE OLSEN

ROBERT GOURDIE

JENNIFER MUNSON

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ABSTRACT

Glioblastoma Multiforme (GBM) is the most common and malignant form of adult brain cancer, with 95% of patients succumbing to the disease within 5 years of diagnosis. An important contributing factor to this poor prognosis is upregulation of the transmembrane protein system x_c- (SXC) found on GBM cells. Approximately 50% of GBM patients have tumors with upregulated levels of SXC, and these patients experience faster disease progression than patients with tumors expressing moderate levels of SXC. SXC is a sodium-independent antiporter and is comprised of a light chain catalytic subunit (xCT) bound to a heavy chain regulatory subunit (4f2hc/CD98) via a disulfide bond. The xCT subunit is responsible for the equimolar exchange of extracellular cystine for intracellular glutamate. Clinical studies have shown areas immediately surrounding the tumor, known as the peritumoral region, reach glutamate concentrations over 100 times that of the normal brain, creating an excitotoxic environment in which neurons cannot survive. In addition to neuronal excitotoxicity, excess glutamate release has also been shown to promote GBM cell invasion, as well as contributing to the clinical presentation of seizures in patients. Moreover, cystine is a component of the antioxidant glutathione, which confers protection to the cells from alkylating therapeutics such as temozolomide (TMZ).

In an effort to identify novel targets that regulate SXC function, I investigated the relationship between SXC and two signaling molecules known to promote GBM progression: CD44 and the epidermal growth factor receptor (EGFR). I experimentally manipulated the CD44-hyaluronic acid (HA) interaction and EGFR to determine if these two signaling molecules were involved in regulating SXC expression and function in two patient-derived GBM cell lines. Experimental data led me to conclude that the tumorigenic potential conferred to GBM cells by CD44 is not related to an interaction with SXC. However, I found that knocking down EGFR led to a significant reduction in SXC expression. These findings are important to the

field, as combinatorial therapies become more actively pursued in clinical trials. Inhibition of EGFR may provide quality of life benefits to patients who suffer from tumor-associated epilepsy through downregulating xCT-mediated glutamate release.

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GENERAL AUDIENCE ABSTRACT

Glioblastoma multiforme (GBM) is an advanced and aggressive form of brain cancer. Incidence of this disease in the United States of America is approximately 3.19 per 100,000 individuals, which translates to more than 13,000 expected annual diagnoses. These tumors arise from genetic mutations that instruct cells to replicate and migrate abnormally. Despite an aggressive medical armamentarium that includes maximal surgical resection, chemotherapy, and radiation, GBM patients have an expected survival period of 12-15 months after diagnosis.

Previous studies have shown that approximately 50% of GBM patients have unusually high expression levels of the System x_c- (SXC) protein. SXC is a protein transporter located at the membrane of GBM cells, and facilitates the exchange of the excitatory neurotransmitter glutamate for the amino acid dimer cystine. SXC exports glutamate out of the tumor cell, where it can then bind to glutamate receptors on surrounding neurons. In the brain, the concentration of extracellular glutamate must be tightly regulated to prevent hyperexcitability of neurons, which may lead to cell death and the induction of seizures. In patients whose tumors highly express SXC, studies have shown that glutamate levels can rise to concentrations over 100 times greater than the levels seen in normal brain tissue. Additionally, glutamate has been shown to stimulate GBM cells to migrate within the brain and establish secondary tumor sites.

The medical and scientific community is justifiably interested in discovering novel methods for regulating or inhibiting SXC-mediated glutamate release. While SXC inhibitors have been identified, clinical studies have determined they are not appropriate for the clinical treatment

of GBM. Thus the focus of this project was to identify novel molecular regulators of SXC. To that end, I explored two signaling molecules that are known to promote GBM pathogenesis: CD44 and the epidermal growth factor receptor (EGFR). I found no evidence to support a role for CD44 in regulating SXC in GBM. However, I was able to determine, through genetic and pharmacologic manipulation of patient-derived GBM cells, that EGFR regulates SXC expression and function. The results of these experiments confirmed EGFR as a key signaling protein involved in orchestrating SXC-mediated glutamate release, and may inform future clinical studies investigating combinatorial therapies for GBM patients.

DEDICATION

I would like to dedicate this dissertation to my parents, Ron and Linda Martin. Getting to the point of completing a PhD would not have been possible without the hard work and sacrifices you have both made for me throughout my life. Thank you for always fostering my creative and scholastic interests, for encouraging my active imagination, and for providing me the freedom to ask questions and explore the answers independently. Mom, thank you for the countless hours you spent helping me with homework, instilling in me a strict work ethic, and for showing me that the extra mile is always worth the effort. Dad, thank you for always being a cheerleader, for letting me dream as big as I dared, and for teaching me that *tough times don't last, but tough people do*.

"For I know the plans I have for you," declares the LORD,

"plans to prosper you and not to harm you,

plans to give you hope and a future."

Jeremiah 29:11

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LIST OF ABBREVIATIONS

AKT	Protein kinase B
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
CAR	Chimeric antigen receptor
CD44s	CD44 standard isoform
CD44v	CD44 variant isoform
CNS	Central nervous system
CSC	Cancer stem cell
EAAT	Excitatory amino acid transporter
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FDA	Food and Drug Administration
GAG	Glycosaminoglycan
GBM	Glioblastoma multiforme
GlcA	D-glucuronic acid
GlcNAc	N-acetyl-D-glucosamine
GSH	Glutathione
HA	Hyaluronic acid
HYAL	Hyaluronidase
K^+	Potassium
kDa	Kilodalton
MGMT	O6-Methyl guanine DNA methyltransferase
MMP	Matrix metalloprotease
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
mTORC2	Mechanistic target of rapamycin 2
Na^+	Sodium
NMDA	N-methyl-d-aspartate

LIST OF ABBREVIATIONS (CONTINUED)

NSC	Neural stem cell
OPC	Oligodendrocyte progenitor cell
OS	Overall survival
PDGF	Platelet derived growth factor
PDX	Patient-derived xenoline
PFS	Progression-free survival
PLA	Proximity ligation assay
PNN	Perineuronal Net
RT	Room temperature
RTK	Receptor tyrosine kinase
S-(4)-CPG	(S)-4-carboxyphenylglycine
SAS	Sulfasalazine
SVZ	Subventricular zone
SXC	System x_c-
TMZ	Temozolomide
VEGF-A	Vascular endothelial growth factor A

CHAPTER ONE: INTRODUCTION

1.1 *Glioblastoma multiforme*

Humans suffer various afflictions of the brain throughout life that include, but are not limited to: neurotransmitter imbalances, physical trauma, neurodegeneration, prions, stroke, and tumors. Of particular interest for this dissertation are brain tumors, which are categorized as cellular masses that behave abnormally due to the accumulation of genetic mutations. Glioblastoma multiforme (GBM) is the most common and aggressive form of adult brain cancer, and claims the lives of thousands of Americans every year [1].

The World Health Organization designates GBM as a stage IV glioma due to the highly invasive and proliferative nature of the cells composing the tumor mass, as well as the rapid disease progression and high likelihood of recurrence that characterizes this disease [2]. Prolonged or excessive radiation exposure is the predominant risk factor for developing GBM, and some additional evidence suggests a genetic predisposition to the disease [3-5], however the majority of GBMs arise from an unknown origin. Epidemiological studies have consistently found a higher incidence of GBM in individuals with Caucasian heritage compared to those of Asian or African descent [6]. Furthermore, males are 1.58 times more likely to develop GBM than females [1], and female GBM patients appear to have a better response to treatment and increased survival time compared to males [7, 8].

GBMs are most often diagnosed after the age of 55 and the current standard of care for patients includes maximally safe surgical resection, chemotherapy, and radiation [9]. Although treatments exist, GBM remains incurable. On average, GBM patients succumb to this disease within 12-15 months after diagnosis, even if they undergo treatment [10]. The percentage of patients surviving 5 years is less than 3%, and only 1% of patients are long-term survivors of 10 years or longer [6, 11]. The current standard of care chemotherapeutic intervention, temozolomide (TMZ), adds only

2-3 months of increased survival time for patients [12]. Clearly, a critical unmet need for targeted, efficacious therapy exists for these patients and warrants further scientific investigation.

Glioblastoma Origins

The name glioblastoma multiforme offers helpful information about the disease itself. The component parts include *glio* + *blast* + *oma* + *multiforme. Glia* is derived the Greek word "γλία", meaning "glue" and refers to the category of central nervous system (CNS) cells known as glia. In medical nomenclature, "blast" typically refers to immature cells. "Oma" is a Greek derivative of "ωμα" and is currently accepted in the medical community to indicate a swelling or tumor [13]. The addition of "multiforme" is a nod toward the intensely varied nature of these tumors. Taken together, the name *glioblastoma multiforme* indicates a tumor comprised of immature glial cells that harbor many different kinds of mutations.

Contrary to the common misconception that the brain is incapable of generating new cells, the brain does house a population of stem cells with proliferative and regenerative capacity, which are largely confined to a region of the brain known as the subventricular zone (SVZ) [14]. These stem-like cells in the SVZ are known as neural stem cells (NSCs) and oligodendrocyte progenitor cells (OPCs). NSCs and OPCs are the primary suspects in gliomagenesis due to their self-renewal and differentiation capabilities. Additionally, these cell populations are in close proximity to the SVZ, where a clinical study found that 93% of gliomas made anatomical contact [15]. Recent studies show support for both cell types as the origin for GBM, and found that NSCs and OPCs with driver mutations are able to migrate away from the SVZ and contribute to gliomagenesis in cortical regions of the brain [16]. However, OPCs are derived from the NSC lineage and harbor the same mutations as their NSC precursor cells, which more clearly supports mutated NSCs as the primary driver of GBM pathogenesis [17]. Studies conducted to understand the evolution of GBM and the

risk factors that increase the likelihood of developing this disease will provide powerful insights to the medical community that will support early detection and intervention.

Approximately 90% of GBMs arise through *de novo* somatic mutations (primary GBM), and only 10% of GBMs develop through the evolution of lower-grade gliomas such as astrocytomas or oligodendrocytomas (secondary GBM) [18]. Although developmentally different, primary and secondary GBMs are morphologically indistinguishable. GBMs have a propensity for invading into deep recesses within the brain, most often along periturmoral secondary structures such as white matter tracts, blood vessels, and the meninges [19]. Despite the well-described invasive nature of GBM, these tumors rarely metastasize out of the brain (0.5%) [20, 21]. This seemingly paradoxical behavior could be a result of any combination of factors, including innate tropism of GBM cells for the CNS environment, lack of adequate escape routes from the brain, and/or increased immune cell surveillance outside of the CNS [20].

Unlike other brain tumor types such as meningiomas or medulloblastomas, GBMs typically grow in the central parenchymal regions of the brain. The majority of GBMs (~95%) are located in the supratentorial region of the brain [22], particularly in the frontal and temporal lobes [23]. The location of primary and satellite tumors in this region is likely a result of interstitial flow and neurotransmitter projections that influence tumor cell migration and behavior [24, 25].

Heterogeneity in glioblastoma multiforme

Upon diagnosis, GBMs are categorized into four subtypes: classical, neural, proneural, and mesenchymal, based on the molecular profile of the tumor [26]. The classification of each tumor into one of the aforementioned subtypes is based on the genetic signature of each tumor and the specific categories of mutations it harbors. While each tumor has a unique combination of mutations, tumors within these subtypes can be stratified by general survival outcome and

therapeutic response [27]. For example, GBMs of the classical subtype tend to be initially responsive to radiation and chemotherapy, an outcome that is largely attributed to O6-Methyl guanine DNA methyltransferase (MGMT) DNA methylation, which prevents the tumor cells from repairing damaged DNA after treatment [26]. GBMs of the neural subtype also show a relatively high response rate to therapy, and appear to have highly similar genetic signatures to nearby neural cells [26]. Indeed, the status of the neural subtype has come into question after transcriptome analysis of single GBM cells revealed a lack of enrichment for the neural subtype, and that these cells are instead marginal non-tumor neural cells [28, 29].

Secondary GBMs frequently fall into the proneural category and occur in younger individuals, which contributes to the longer survival times observed with proneural GBMs compared to the more aggressive mesenchymal subtype [28]. Approximately 30-49% of GBMs are considered mesenchymal, and these tumors tend to be more invasive and more resistant to radiation therapy than other GBM subtypes [27]. Interestingly, GBMs often transition from the proneural subtype to the mesenchymal subtype upon recurrence after radiation therapy [28], which may indicate that these cells may be selected for when challenged with treatment. However, the predictive and therapeutic value of GBM subtyping should be considered with caution, as single cell RNA-sequencing studies confirmed the presence genetic signatures of all four subtypes within the same tumor, and even markers of multiple subtypes within the same cell [30]. Regardless of the GBM subtype identified, patients will most often undergo a treatment regimen of concomitant radiation therapy and TMZ after surgical resection [31]. The inherent heterogeneity of GBMs presents a myriad of clinical challenges [32], although there are several avenues of clinical development that are currently being explored to identify efficacious therapies for GBM.

Current and Investigational Therapeutics

As a direct result of the brain's placement in the confines of the skull and the delicate nature of the cognitive and tactile functions controlled by cortical tissue, clinical presentation of GBM is often sudden and severe, ranging from sensory deficits, headaches, and cognitive impairments to seizure induction and loss of bodily control [33]. The primary method for detecting GBM is through magnetic resonance imaging (MRI) [34] where GBMs appear as bright lesions surrounded by edema on T2-weighted MRI images. After the identification of an abnormal mass, surgical resection will follow in most cases, and a biopsy of tissue undergoes histopathological analysis to determine genetic markers and cellular abnormalities indicative of brain tumors [35]. Specifically, tumor specimens are analyzed for signs of proliferation, structural abnormalities of the nucleus and cytoskeleton, microvascular hyperplasia, and necrosis that characterize the presence of advanced glioma [36]. Genetic mutations suggestive of a GBM diagnosis include amplification of the epidermal growth factor receptor (EGFR) and platelet derived growth factor (PDGF), presence of the EGFRvIII mutant, homozygous deletion of phosphatase and tensin homolog, and TP53 mutation [37]. Tumor progression and treatment response are routinely assessed by continued MRI imaging throughout the duration of clinical intervention [38].

GBM is an inherently difficult disease to treat for several reasons. GBMs are characterized by diffuse invasion of tumor cells throughout the brain, making complete surgical resection virtually impossible. Additionally, the blood-brain barrier (BBB) presents a significant obstacle to therapeutics administered through oral or intravenous routes in reaching CNS cancer cells. Nevertheless, treatment options do exist that attempt to slow the progression of the disease, and novel compounds or treatment modalities are in continual development. While not completely comprehensive, the goal of this section is to highlight the major classes of therapeutics currently

available or under clinical investigation for the treatment of GBM. The following sections will also shed light on novel protein interactions, altered or unappreciated signaling pathways, and the environment of tumor cells that all contribute to GBM pathogenesis. In this way, we approach the problem with an eye towards uncovering novel mechanisms that can be exploited for the development of chemotherapeutics and other treatments for GBM patients.

The standard of care includes surgery, chemotherapy and radiation. Upon GBM diagnosis, surgery is the first treatment step. Neurosurgeons identify the precise location of the tumor mass through MRI and subsequently perform maximally safe surgical resection of the tumor. Several studies have shown that the extent to which the tumor mass is removed is correlated with longer progression-free survival (PFS) and overall survival (OS) [39]. Even with advanced preoperative planning and imaging techniques that allow the surgeon to navigate through the delicate tissue, surgical resection is not curative and an orthogonal approach to GBM treatment is required. After the cranium has healed from surgery, patients will undergo involved-brain radiotherapy over the course of several weeks with an administered dose of up to 60 Gray. Improvement to radiation treatment in GBM is always being explored, with investigational attempts of brachytherapy and 131-I-labeled antibodies within the resected tumor cavity during surgical resection showing modest success in early clinical trials [40].

Temozolomide (TMZ, brand name Temodar) is the current standard of care chemotherapeutic for GBM patients. TMZ is an orally administered prodrug that functions by adding methyl groups to guanine and adenine residues within GBM cell DNA, which compromises the DNA and inhibits cellular replication [41]. Administration of TMZ typically begins 6 weeks following surgical resection in combination with radiation, and subsequent maintenance cycles of TMZ may be prescribed depending on cancer progression metrics [31]. Combinatorial TMZ and

radiation treatment provided an increased survival benefit of 2.5 months when compared to patients who received only radiation therapy [12]. Despite the meager survival benefit offered by TMZ treatment, it has remained the gold standard GBM chemotherapy since its approval for newly diagnosed GBM in 2005, primarily because it is well tolerated and able to cross the BBB due to its lipophilic nature [42]. Unfortunately, GBM cells have been shown to acquire resistance to TMZ following treatment. This resistance is most likely a result of enhanced DNA repair mechanisms, the presence of renewable cancer stem cells (CSCs), and genetic mutations that allow DNA replication to bypass checkpoints, among other factors in GBM [42].

Nitrosourea therapy (carmustine and lomustine) are similar to TMZ in that they are both alkylating agents that inhibit RNA and DNA synthesis in the remaining GBM cells after surgery [43]. Carmustine is delivered as small wafers that are implanted within the tumor cavity after surgical resection, while lomustine is administered orally [44]. In a phase III placebo controlled study, GBM patients who received carmustine wafers had a survival advantage of 2.3 months, which was significant enough to warrant Federal Drug Administration (FDA) approval in 2003 [43, 45]. Adverse events associated with use of nitrosoureas included brain necrosis, blood clots, seizures, and lymphopenia [43-45]. A meta-analysis found an increased risk (42%) of adverse events after carmustine treatment compared to the control group, which overpowered the slight benefit to overall patient survival [46]. Nevertheless, individual studies regarding the safety and efficacy of carmustine wafers have shown a modest (yet significant) benefit [47].

Angiogenesis inhibitors such as bevacizumab (Avastin) were once thought to be prime candidates for GBM treatment due to the highly vascularized nature of these tumors. Bevacizumab is a monoclonal antibody that binds to vascular endothelial growth factor A (VEGF-A), thereby hindering its ability to bind to the VEGF receptor and inhibiting growth of new blood vessels to

the tumor [48]. The results of two phase III, randomized, placebo controlled clinical trials in patients with newly diagnosed GBM indicated that bevacizumab, in conjunction with standard of care TMZ and radiotherapy, increased PFS, but not OS [48, 49]. Bevacizumab treated patients initially showed a dramatic reduction in cerebral edema, but ultimately the drug offered insufficient clinical benefit based on pre-specified endpoint criteria [50]. The modest PFS benefit was concomitant with a higher incidence of adverse events including bleeding, cardiac issues, impaired wound healing, lymphopenia, and fatigue, among others [48, 49]. Several other angiogenesis inhibitors similarly failed in GBM clinical trials [51-54]. Despite failure to meet the primary endpoint criteria in two clinical trials, bevacizumab was approved for the treatment of recurrent GBM in 2009, after it was determined that it conferred a significant benefit to patients by way of reducing peritumoral edema and radiation associated necrosis that fall outside of PFS and OS criteria [55].

Immunotherapies are still in the investigational stage for GBM and include antibody-mediated cell cycle checkpoint inhibitors, chimeric antigen receptor (CAR) T-cell therapy, and vaccines [56]. The GBM tumor microenvironment is typically characterized by immunosuppressive cytokines, tissue hypoxia, suppression of T-cell proliferation/activation, and the presence of tumor-associated macrophages, all of which contribute to the tumor's ability to evade immune detection and destruction [57, 58]. Therapies that utilize monoclonal antibodies, such as pembrolizumab and nivolumab block receptors that allow GBM cells to evade detection from surveilling immune cells. These therapies are currently in early clinical trials for GBM. However, most of available clinical trial data does not demonstrate that these treatments confer any significant survival benefit, possibly due to the interference of corticosteroid treatment on the immune system [59, 60].

CAR T-cell therapy involves harvesting T-cells directly from the patient and then genetically altering them to promote expression of CARs that are specific to antigens presented on tumor cells. After transfusion back into the patient, the CAR T-cells will eliminate only cells that present tumor-specific antigens [61]. For example, the EGFR mutant EGFRvIII is an attractive target for CAR design because it is expressed on GBM cells, but not normal cells, and thus would specifically direct immune cells to attack tumor cells [62]. While still in the early stages of clinical trials, clinical outcome for GBM patients after CAR T-cell therapy has not offered much promise, although these therapies appear to have strong safety and tolerability profiles [63]. Several limitations for this therapy have been identified, and third generation CAR T-cells are currently being designed with enhanced capacity for survival, immunological activity, and target specificity [64, 65]. Due to the innate heterogeneity of GBMs, designing CAR T-cells that target multiple antigens within the same tumor mass is likely necessary to achieve clinical benefit [66].

GBM vaccines are another personalized medicine approach to GBM treatment that shares similarities to CAR T-cell therapy. While vaccines are traditionally administered in a prophylactic capacity, they can also be applied to initiate anti-tumor immunogenicity. GBM vaccines are composed of a patient's modified immune cells or manufactured peptides that are designed to react to specific antigens expressed on GBM cells in order to provoke an immune reaction against those cells [67]. A phase II clinical trial using an EGFRvIII-targeted peptide showed promising results, with vaccinated patients experiencing significantly longer PFS and OS than patients in the control group [68]. Phase III studies have had mixed results, with one being terminated early due to futility of treatment [69], and another showing a subset of vaccinated patients surviving past 40 months [70]. However, it is difficult to tease apart equivalent trial findings because control arms and antigenic targets differ between trials [56]. Antigenic targets other than EGFRvIII are also under

investigation, which may prove more efficacious [71-74]. Although generally considered safe, the obstacles for this therapy remain in circumventing the immunosuppressive environment of GBM, tumor heterogeneity, ensuring targeted delivery, and the potentially confounding use of steroids during treatment [75], which will be addressed in future trials with advanced vaccine development.

Oncolytic virus therapy is considered an additional form of immunotherapy whereby tumor cells are specifically infected with a virus engineered to undergo conditional replication within cancer cells, destroying the marked cell population [56, 76]. Like the aforementioned vaccines and CAR T-cell therapy, the viral particles are designed to target cells with surface antigens that are unique to the tumor cells [67]. Additional non-lytic viral therapies are being explored that deliver therapy into the tumor cells once they are infected [77]. An open label, phase II/III clinical trial using a retroviral vector in combination with chemotherapy for GBM failed to provide a survival benefit compared to the standard of care [78]. However, data from a phase I clinical trial using recombinant poliovirus delivered directly into the tumor had promising results, with 21% of patients surviving past 24 months (compared to previously reported 13% with standard of care) [79], and two patients surviving past 60 months after viral infusion [80]. A phase II poliovirus study is currently underway and expected to be completed in 2023 [81]. With these encouraging results and the relatively rare incidence of severe adverse events in response to viral therapies, further investigation of this treatment modality for GBM is warranted [82].

Optune (tumor treating fields) is a wearable device that sends low intensity electric fields through transducers on the scalp to GBM cells. Mechanistically, these electric fields are thought to impair mitosis and induce cell death [83]. Optune was approved by the FDA in 2011 and in 2015 for GBM patients with recurrent and newly diagnosed GBM, respectively [31]. The unprecedented PFS and OS benefit to patients treated with Optune and TMZ compared to patients

receiving only TMZ led to a recommendation to terminate the phase III trial early [84]. Optune users experience milder side effects compared to chemotherapy, and this treatment contributed to a superior quality of life metric for patients compared to those receiving only chemotherapy [83]. Optune therapy is now undergoing clinical trials in combination with approved or candidate treatments to explore the possibility of synergistic benefits [85, 86].

Receptor tyrosine kinase (RTK) inhibitors are small molecule treatments that typically bind irreversibly to the adenosine triphosphate (ATP) binding pocket of RTK proteins such as EGFR, VEGF, fibroblast growth factor receptor, and the PDGF receptor [50]. Second and third generation RTK inhibitors selectively bind to regions adjacent to or removed from the ATP binding site of these proteins, but all function to prevent the necessary conformational change that propagates downstream signaling pathways that enhance cell survival, proliferation, angiogenesis, migration, and invasion [87]. Despite showing efficacy in solid tumors of the periphery, RTK inhibitors have had a disappointing performance in GBM [88]. Postulated reasons for these failures include difficulty in targeted delivery, the genetic heterogeneity of GBMs, acquired mutations in the ATP binding site of the RTK, interference by anti-epilepsy medication often taken by GBM patients, and redundant signaling pathways that lead to tumor propagation [87-89]. Because RTK inhibitors have been unsuccessful in the treatment of GBM, inhibition of downstream targets of RTKs are currently being investigated through ongoing clinical trials and have thus far demonstrated tolerable safety profiles [90, 91].

As the abundance of clinical trial data show, the dearth of newly FDA approved treatments for GBM in the past 10 years is not for a lack of effort. GBM in particular is replete with physical, chemical, and genetic barriers that significantly complicate drug development. A common theme found among the disappointing trial results is the fact that GBMs often possess (either innately or

through Darwinian acquisition) compensatory mechanisms that allow the tumors to continue proliferating even in the face of theoretically robust therapies. It is clear that a multidimensional approach is the best path forward for combatting this disease. Encouragingly, newer treatments like immunotherapies and Optune appear to have manageable side effects and few patients experience severe adverse events. As of March 2021, the natural killer cell therapy CYNK-001 was granted fast-track approval by the FDA for treatment in patients with recurrent GBM based on current phase I clinical trial data [92]. Studies like this one provide hope that new and creative treatments utilizing immunotherapy are on the horizon, though expanded clinical trials are needed and FDA approval could still be years away. While the current outlook for the clinical management of GBM remains rather bleak, many groups in the private, governmental, and academic sectors are expending vast amounts of resources to identify novel targets for GBM while also devising inventive ways to exploit the weaknesses of individual tumors. Since no two GBM tumors are the same, the personalized medicine tactics illustrated by CAR T-cell and vaccine therapies offer heretofore nonexistent tailored medical strategies that have the potential to advance our current armamentarium against GBM.

1.2 *Maladaptive excitatory neurotransmission in glioblastoma multiforme*

Brain homeostasis is required for appropriately functioning neural and glial cells, and is contingent upon the masterful coordination of a wide variety of transporters and signaling molecules that ensures the physiological environment of brain cells is within an acceptable range. Glutamate is the most abundant excitatory neurotransmitter of the CNS, and is essential for neural processes such as synaptogenesis, intercellular communication, cognitive functions, and cellular metabolism [93, 94]. Glutamatergic neurotransmission is one of the key mechanisms through

which neural cells communicate with each other, and occurs through a process whereby glutamate molecules are packaged into small organelles enclosed by a lipid bilayer, known as vesicles. These vesicles are then transported and docked to the membrane of a pre-synaptic neuron, where approximately 8,000 glutamate molecules are released from each vesicle into the extracellular space where they will bind to and activate glutamate receptors on post-synaptic neurons, thereby propagating excitatory signaling cascades [95, 96]. However, extracellular glutamate concentrations must be tightly regulated so as to not exceed 1-2 μM, which leads to pathological conditions including seizures, headaches, or neurodegeneration [94].

Aberration in glutamatergic homeostasis is well characterized in GBM, and can be attributed to multiple factors. Glioma cells have a higher resting membrane potential than healthy glial cells, resulting from mislocalization of inward rectifying potassium (K^+) channel proteins to the nucleus and intracellular membranes of GBM cells, rather than the plasma membrane [97, 98]. The mislocalization of these channels leads to an imbalance of extracellular K^+ ions and an inappropriate sodium (Na^+) gradient, ultimately resulting in the inactivation of Na^+-dependent glutamate transporters [98]. The proper localization and function of inward rectifying potassium channels on healthy glial cells is integral to their purpose of buffering K^+ concentration in the extracellular space, which allows surrounding neurons to maintain a low resting membrane potential of approximately -70mV [99]. Improper glial cell K^+ buffering leads to hyperexcitable neurons, potentially inducing severe neuronal damage or excitotoxic cell death. Neuronal excitotoxicity occurs when neuronal firing is deregulated due to excessive glutamate exposure, causing a lethal influx of calcium into the cell and the induction of apoptosis [100, 101].

Extracellular Na^+ and K^+ imbalance impairs the functionality of Na^+-dependent transporters, particularly the family of excitatory amino acid transporters (EAATs). These

transporters are abundantly expressed on glial cells and mediate the rapid uptake of excess glutamate molecules from extracellular and extrasynaptic spaces, which helps prevent excitotoxicity in nearby neurons [98]. Studies have also shown a lack of EAAT expression and/or mislocalization of EAATs in GBM cells, which prevents these cells from sequestering extracellular glutamate as their normal glial counterparts do [102, 103]. To the contrary, GBM cells actually expel large quantities of glutamate into the extracellular space [104], through a mechanism that will be discussed in the following section. Furthermore, proteases released from GBMs degrade the insulating perineuronal nets (PNNs) of nearby GABAergic interneurons, compromising the ability of these inhibitory cells to curb the enhanced excitatory signaling cascades derived from the aforementioned aberrations that promote glutamatergic excitotoxicity [105].

In summary, GBM cells contrive an environment where normal ion concentration gradients are disrupted, and an imbalance between excitatory and inhibitory signaling develops. The inability of peritumoral glial cells to regulate extracellular ion concentration results in hyperpolarized excitatory neurons and reduced firing capacity of inhibitory interneurons [105]. This unchecked excitatory glutamatergic signaling acts as the kindling for surrounding neuronal cell death and the induction of tumor-associated seizures. This is unsurprising given the ability of GBM cells to impair the ability of glial cells to remove glutamate from the extracellular space, while simultaneously releasing glutamate themselves.

1.3 *System x_c-*

A large percentage of the glutamate released by GBM cells can be attributed to the system x_c- (SXC) transporter, which remains a fully functional source of non-vesicular glutamate release

on GBM cells despite the many irregularities present in the GBM environment [106]. System x_c- is a heterodimeric plasma membrane-bound amino acid transporter that mediates the equimolar exchange of extracellular cystine for intracellular glutamate [107]. The system x_c- complex is comprised of a heavy-chain subunit (CD98/4F2hc) and a catalytic subunit (xCT) that is responsible for amino acid transport [108], illustrated in Figure 1.1. CD98 functions as a chaperone protein for several different amino acid transporter proteins, and is required for the correct trafficking of xCT into the plasma membrane [109]. The human xCT protein is comprised of 501 amino acids that span 12 transmembrane domains, with both N and C termini located intracellularly [110]. The xCT protein belongs to a family of solute carrier proteins that function in a sodium independent, chloride dependent, manner [108]. The molecular weight of xCT is approximately 55 kilodaltons (kDa) and the protein is transcribed from the *SLC7A11* gene found on chromosome 4q28.3 [110]. The importance of the xCT protein in cellular survival is evidenced by its highly conserved nature across vertebrate species [107]. Once transported to the cytoplasm, the amino acid dimer cystine is quickly reduced to two cysteine molecules, which are then used as building blocks for the synthesis of glutathione (GSH), an important antioxidant used to counteract the effects of damaging reactive oxygen species (ROS) that challenge cells [111]. In exchange for cystine, xCT exports glutamate out of the cell and into the extracellular space [108].

System x_c- is present on many different cell types throughout the body and has been implicated in various diseases including drug addiction [112], epilepsy [113], neurodegenerative disorders including Alzheimer's Disease and Parkinson's Disease [114], and brain tumors [106, 115]. Notably, but unsurprisingly, the aforementioned diseases all negatively affect the CNS. This is largely because aberrant xCT activity leads to dysregulated glutamate signaling in the synaptic cleft and surrounding areas that disrupts functional neurotransmission and nearby astrocytes.

Maladaptive glutamate signaling as a result of xCT overexpression is evident in GBM. Approximately 50% of GBM patients have upregulated levels of xCT, which leads to high extracellular glutamate concentrations and creates an excitotoxic environment for peritumoral neurons [115]. This increase in extracellular glutamate impairs the ability of neurons to transmit signals in a synchronous fashion and increases the risk that patients will develop tumor-associated epilepsy [116]. Glutamate uptake experiments using human GBM cell lines was reduced by approximately 50% in the absence of sodium, indicating that half of glutamate transport is a result of sodium-independent transporter activity, namely that of xCT [103]. Additionally, autocrine and paracrine glutamate signaling resulting from xCT activity has been demonstrated to promote migration and invasion of GBM cells by inducing calcium oscillations that stimulate cellular motility [117]. As if the expelled glutamate was not detrimental enough, GBM cells utilize imported cystine for downstream GSH synthesis, which confers both resistance from alkylating chemotherapeutic agents such as TMZ [118] and protection from endogenous ROS products resulting from the increased metabolic load of many cancer cells, including GBM [119]. To illustrate this point, studies with GBM cell lines overexpressing xCT had IC_{50} values 6-fold higher compared to control cells after TMZ treatment, in addition to significantly greater production of GSH and mitochondrial activity [118]. Furthermore, xCT-mediated glutamate release has been shown to indirectly promote the proliferation and activation of immunosuppressive T-regulatory cells, which allowed glioma cells to evade immune detection and decreased patient response to anti-angiogenic treatment [120]. System x_c- has proven to be a salient protein involved in the progression of multiple diseases, and thus its regulation from both endogenous and pharmacological sources has garnered much interest.

Modulation of system x_c-

Multiple inhibitors of system x_c- have been discovered and studied in the context of GBM. Notably, the prodrug sulfasalazine (SAS), which was first approved for Chron's Disease and crosses the BBB, is an allosteric antagonist of xCT, and inhibits the exchange of glutamate and cystine across the plasma membrane [121]. SAS is comprised of 5-aminosalicylic acid linked to sulfapyridine via an azo bond, both of which have been demonstrated to be active moieties [122]. *In vivo* studies have shown that SAS effectively reduced peritumoral glutamate concentrations and seizure incidence in murine models of GBM [123, 124]. In a clinical pilot study, acute oral administration of SAS resulted in reduced peritumoral glutamate concentrations in patients when measured by magnetic resonance spectroscopy [115, 125]. Patients with the highest xCT expression levels (as determined through chromogenic staining of tumor biopsy tissue) showed the greatest response to SAS. However, an increase in peritumoral glutamate levels was observed 24 hours post drug administration, suggesting the positive effects of SAS are short-lived [115]. Additional clinical studies have examined SAS as a GBM therapeutic, but the overall short bioavailability of the drug and generalized adverse reactions have thus far precluded it from becoming a standard of care therapeutic for GBM patients [126, 127].

In addition to SAS, another compound known as (S)-4-carboxyphenylglycine (S-(4)-CPG) has proven efficacious in inhibiting xCT-mediated glutamate release. *In vitro* studies using multiple glioma cell lines showed that incubation with 100 µM S-(4)-CPG resulted in a dramatic drop in extracellular glutamate concentrations, and that S-(4)-CPG was the most potent inhibitor of xCT activity compared to other phenylglycine derivatives [104]. Moreover, both SAS and S-(4)-CPG treatment resulted in a significant decrease in glioma cell growth [124]. *In vivo* experiments using glioma-implanted rats demonstrated that intrathecal administration of S-(4)-

CPG resulted in significantly reduced peritumoral edema, slowed progression of neurological deficits, and prolonged survival compared to vehicle-treated rats, despite no observed difference in overall tumor volume [128]. To date, S-(4)-CPG has not been utilized in any human clinical trials. This is likely due to the activity of S-(4)-CPG as a competitive antagonist of metabotropic glutamate receptors [129], which would cause deleterious off-target effects in CNS neutotransmission in addition to inhibiting xCT-mediated glutamate release. Nevertheless, inhibition of xCT clearly has therapeutic value, as demonstrated by the findings of several *in vitro* and *in vivo* studies.

Several studies have recently brought to light new and important information regarding the endogenous regulation of *SLC7A11*/xCT at the transcriptional, environmental, and protein levels in many cancer-types, including GBM [110]. At the messenger RNA (mRNA) level, several well-defined transcription factors bind to and regulate the promoter region of the *SLC7A11* gene, including Nrf2 (activator) in response to oxidative stress [130], ATF4 (activator) in response to nutrient deprivation [131], Oct1 (activator) [132], BRD4 (activator) [133], BAP1 (suppressor) [134], and P53 (suppressor) [135]. All of the aforementioned transcription factors work in concert with one another, responding to signals of oxidative stress and thus inducing mechanisms by which the cell gains greater access to GSH. Translation of *SLC7A11* mRNA into functional protein is inhibited in many cancers through micro-RNAs such as miR-26B and miR-375, which bind to the 3' untranslated region of mRNA [136, 137]. Post-translational phosphorylation of xCT at Ser-26 by the mechanistic target of rapamycin 2 **(**mTORC2) and protein kinase B (AKT) inhibit xCT activity [138]. These examples illustrate the complex nature of activating or suppressing xCT expression and function, and highlight the importance of considering the context of cancer subtype and cellular environment in which each of these studies were conducted.

The xCT protein is highly sensitive and responsive to cues in the extracellular environment. Induction of oxidative stress in xCT-expressing cells by the addition of buthionine-sulfoximine [139], hydrogen peroxide [140], diethyl maleate [141], or through glucose starvation [142] led to increased xCT expression and activity in response to depleted levels of GSH. To our knowledge, the half-life of the xCT protein has not yet been established, yet a few studies provide indirect clues that it could be 16-24 hours [143, 144]. However, this timing would be a function of the cellular environment and antioxidant needs at any given time.

In conjunction with environmental and transcription factors, protein binding partners of xCT have been shown to regulate the expression and function of xCT. Studies on this topic demonstrated a stabilizing interaction between the intracellular domain of the epidermal growth factor receptor (EGFR) and amino acid residues 45–470 of xCT in GBM, which promoted xCT-mediated glutamate release in glioblastoma cells [143]. Multivariate analyses identified EGFR amplification as a significant factor in predicting if glioma patients would experience preoperative seizures [145], which is a salient and interesting observation considering the aforementioned relationship between EGFR and xCT, as well as the fact that seizures are also associated with upregulated xCT expression.

The CD44 variant isoform that includes variant exons 8-10 (CD44v8-10) has been demonstrated to interact with and provide functional stabilization of xCT on the plasma membrane of gastrointestinal cancer cells [146]. In lung cancer, upregulation of the epithelial regulatory splicing protein (ESPR1) leads to preferential splicing of the variant CD44v8-10, accompanied by an increase in xCT expression and GSH levels in metastatic cells. This confers added protection from ROS and other stressors to these cells as they establish secondary tumor sites [147]. Furthermore, experiments in human lung carcinoma and neuroblastoma cells showed that the

ubiquitinase OTBU1 interacts with and stabilizes xCT in a CD44-dependent manner by forming a tri-protein complex, with OTBU1 binding to the N-terminus of xCT and CD44 binding to the C-terminus [144]. However, it is worth noting that this interaction was not specifically demonstrated in GBM. Both EGFR and CD44 are demonstrated regulators of xCT in several cancers, and more specific information about each of these important signaling proteins in the context of GBM is discussed in greater detail in the following sections.

1.4 *CD44 and hyaluronic acid*

CD44 is a single-pass, ubiquitously expressed transmembrane glycoprotein that is best known for its role in cellular adhesion and motility, proliferation, tissue development, wound healing, angiogenesis, and tumor invasion [148-150]. The *CD44* gene is located on chromosome 11p13 and is composed of 20 exons [149]. Exons 1-5 are spliced together with exons 16-20 (S1-5, S6-10) to create the standard isoform of CD44 (CD44S, CD44H), while ten variant exons (6-15, V1-10) are interchangeably spliced together with the standard exons to form alternative isoforms of CD44 (CD44v) and is illustrated in figure 1.2A [151]. CD44S has a molecular weight of ~85 kDa, and the molecular weight of variant CD44 isoforms can be up to 200 kDa [149]. The CD44 protein has three domains: the cytoplasmic domain, the transmembrane domain, and the extracellular domain. Expression of variant exons in CD44 adds amino acids to the either the cytoplasmic tail or more frequently, the proximal stem region of the extracellular domain. These amino acid additions alter protein conformation and the binding motifs for ligands as well as sites for post-translational modifications such as N- and O- linked glycosylation [152, 153], and is illustrated in figure 1.2B. CD44S most commonly undergoes N-glycosylation, while CD44v isoforms tend to be more heavily O-glycosylated [154]. This alternative splicing creates many

isoforms of CD44, each with unique binding and signaling properties that can be utilized differentially depending on the unique needs of the cell [155]. Unsurprisingly, neoplastic cells of solid tumors also utilize the differential properties of CD44 isoforms to their advantage, including GBM cells [156]. For example, CD44S appears to be the dominant form of CD44 present in GBM and other brain tumor types, although other CD44 variants have been observed in GBM as well as lower grade gliomas [157, 158]. Of note in GBM is the absence of the variant CD44E (CD44v8-10), which contains variable exons 8-10 and is primarily found in epithelial tumors such as gastrointestinal cancers [143, 159].

CD44 has been implicated as a marker of cancer-initiating cells and cancer stem cells in multiple solid tumors because of the self-renewal capabilities observed in cells that express high levels of CD44 [152, 160]. CD44 is frequently overexpressed in GBM cells [161], and has been implicated in conferring therapeutic resistance and regenerative potential to osteosarcoma cells [162], breast cancer cells [163], and GBM cells [156, 164]. Evidence supporting the classification of CD44 as a cancer stem cell marker come from studies demonstrating that the transition from CD44v to CD44S promotes epithelial-mesenchymal transition (EMT) in cancer cells, an important feature of metastatic tumors as it enhances their ability to migrate, invade, and proliferate in other tissues [165]. Activation of CD44 stimulates the PI3K/AKT signaling pathway to promote cell survival and EMT [166], as well as activating matrix metalloproteases (MMPs) to aid in the process of tissue remodeling necessary for tumor invasion and metastasis [149]. However, there lacks complete agreement within the literature about whether or not CD44 should be considered a glioma stem cell marker. The majority of evidence against CD44 as a stem cell marker lies with a study showing an inverse relationship between CD44 and other stem cell markers such as NANOG, OCT4, and Nestin in GBM cells after TMZ treatment [167].

Regardless of its formal classification as a cancer stem cell marker, substantial evidence suggests that CD44 plays a role in tumorigenesis. Proteolytic cleavage of CD44 creates soluble extracellular and intracellular domain fragments [168]. MMPs mediate the cleavage of the extracellular domain of CD44 in response to cues such as calcium influx or protein kinase C activation [169]. The free soluble extracellular domain promotes the canonical functions of CD44 such as cellular adhesion, migration, and invasion [161]. Shedding of the soluble CD44 ectodomain stimulates cleavage of the truncated CD44 intracellular domain by presenilin-dependent γ-secretase [168, 170]. The free intracellular domain is then translocated into the nucleus where it acts as a transcription factor to promote transcription of CD44 itself, as well as other tumorigenic proteins in a positive feedback loop [149, 168].

A critical requirement for metastatic tumors is the ability to obtain nutrients once they have established a secondary site. Tumors achieve this by generating new blood vessels, a process known as angiogenesis [171]. CD44 has been implicated angiogenesis of multiple cell types [172, 173]. In a mouse model of melanoma, CD44-null mice had significantly reduced tumor vascularization compared to CD44 wild type mice, and the vessels that did form in the CD44-null mice were abnormal [174]. GBMs are highly vascularized tumors, and a significant subset of tumors rely on newly formed blood vessels as a pathway for invasion [175]. CD44 promotes GBM survival by supporting angiogenesis, which not only enables cancer cells to obtain sufficient nutrients, but also provides a structural route for invasion [172, 176, 177].

Hyaluronic acid (HA, also known as hyaluronate or hyaluronan) is the primary binding ligand for CD44 [150]. HA is a negatively charged glycosaminoglycan (GAG) that comprises a large percentage of the extracellular matrix (ECM) and is essential for supporting tissue structure, providing protection from compressive forces against tissues, cellular water retention,

homeostasis, and intercellular communication [178, 179]. Three hyaluronic acid synthase enzymes (HAS1, HAS2, and HAS3) at the plasma membrane synthesize HA by assembling repeating units of N-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA) through glycosidic bonds and then extruding the completed polysaccharide into the ECM [180, 181]. The HAS molecules assemble linear HA polysaccharide chains that can reach sizes up to $2x10^6$ Daltons [180]. HA is synthesized enzymatically at the cell membrane rather than in the Golgi apparatus like other GAGs, and as a result it is the only non-sulfated GAG [182]. HA is also unique in that it forms noncovalent bonds with proteoglycans rather than covalent bonds between -serine-glycine- residues of a proteoglycan and a linker tetrasaccharide on the GAG [183, 184].

While HA binds to CD44 with the highest specificity, it is also able to form non-covalent bonds with other proteoglycans such as the receptor for hyaluronan-mediated motility, LYVE-1, and hyaluronectin, among others [159, 185]. While the affinity for HA on a single CD44 protein is relatively low, this interaction is strengthened by multivalent interactions between large HA polymers binding to many individual CD44 protein clusters on the cell membrane [186]. In humans, HA is degraded by a 6-member family of enzymes, which include four endogenous hyaluronidases (HYAL1-4, PH20, HYALP1) [180]. Human HYALs cleave the β-1,4 glycosidic linkages between GlcNAc and GlcA and create short HA oligomers that have different signaling properties than the native high molecular weight form [187]. HYAL also appears to play an important role in tumor cell invasion and angiogenesis in multiple cancer types, including GBM. In one study, all four GBM cell lines tested expressed mRNA for HYAL, whereas cells from healthy brain regions expressed undetectable levels of *HYAL* mRNA [188]. Multiple studies have shown that metastatic tumor cells (including GBM) express higher levels of HYAL than stationary

cells- illustrating why the HYAL family was first coined as “spreading factors” after their initial discovery in the early 1900s [188-190].

Extensive investigation of HA has revealed its capabilities as a diverse signaling molecule. HA has a documented involvement in pathways such as RAS, MAPK, and PI3K/AKT, which initiate cellular processes such as cell motility, invasion, adhesion, growth and differentiation [185, 191]. The precise effects of HA depend on receptor expression of the cell it interacts with, as well as the location of the cell within the body, and perhaps most importantly, the size of the HA polymer [180]. It is generally understood that high molecular weight HA (≥100 kDa) is involved in homeostatic functions by inhibiting cell proliferation, inflammation and migration, whereas low molecular weight HA (≤500 kDa) is involved in pathological functions by promoting inflammatory signaling, angiogenesis, cell motility, and proliferation [192]. Low molecular weight HA fragments can also bind to CD44 and disrupt the interaction between CD44 and high molecular weight HA, further obstructing normal signaling pathways [193]. The observed differences in cellular response is a function of an altered ability of HA polymers of varying sizes to interact with their receptors, allowing for differential outcomes even though the molecular composition of HA remains constant [193]. CD44 variants also have distinct HA-binding capabilities [194], highlighting the diversity of HA as a signaling molecule in healthy and pathologically transformed tissues. The functional plasticity of HA is easily exploited by cancer cells, and is a primary reason why the presence of high concentrations of HA of various sizes in solid tumors and the surrounding stroma is correlated with worse prognosis for patients [195], overall tumor aggressiveness [181, 196], and treatment resistance [197]. HA may also be a viable tumor marker, as HA levels in the sera of patients with metastatic cancers of the bladder, prostate, and lymphatic system were significantly higher than those of patients without metastatic disease [198].

1.5 *The Epidermal Growth Factor Receptor*

The epidermal growth factor receptor (EGFR)/HER1/ErbB1 is a single-pass, transmembrane protein comprised of an extracellular binding motif, a short, hydrophobic transmembrane domain, and a cytoplasmic tail that mediates tyrosine kinase and C-terminal phosphorylation activity [199], illustrated in Figure 1.3. The extracellular region of EGFR is divided into four domains: I, II, III, and IV, of which I and III are principally responsible for ligand binding [200]. The human *EGFR* gene is located on chromosome 7p11.2 and the EGFR protein is ~170 kDa [89, 201]. EGFR is a well-described tyrosine kinase receptor activated through binding to its principal ligand, epidermal growth factor (EGF), in addition to other activating substrates [202, 203]. EGFR has many tyrosine phosphorylation sites, which are mostly located on the cytoplasmic tail domain [204]. In particular, phosphorylation of tyrosine 845 in the kinase domain is important for survival and proliferation of GBM cells [204-206]. Activation of EGFR leads to receptor dimerization, auto or transphosphorylation, and activation of downstream signaling through the MAPK, PKC, RAS, Src, STAT3/5, mTORC, and PI3K/AKT pathways [89]. These pathways promote cellular proliferation, survival, motility, and differentiation, which is why EGFR is essential to the proper growth and maturation processes of cells and tissue. The importance of EGFR is exhibited by murine studies showing that knockout of EGFR during gestation or shortly after birth is lethal due to developmental defects, particularly in neural and cardiac tissue, as well as epithelial tissue comprising the skin and lungs [207, 208].

EGFR is well known for its role in oncogenesis, particularly in lung cancer, breast cancer, and GBM [209]. EGFR is part of a larger family of receptor tyrosine kinases (including ErbB2, ErbB3, and ErbB4), all of which become activated in response to growth factor binding and through the formation of homodimers or heterodimers. EGFR heterodimer complexes are less stable than

homodimers and have an impaired ability to recruit endocytic machinery to the complex, which results in weaker, yet prolonged EGFR signaling that is enhanced in oncogenesis [210]. Propagation of EGFR signaling cascades relies on a conformational change of EGFR that reveals the binding pocket for ATP at lysine 745 on the cytoplasmic domain [211]. Inhibiting the ability of ATP to successfully dock to its binding site on the EGFR protein, and thus preventing downstream survival and proliferation signals, is the basis for cancer therapeutics such as erlotinib and gefitinib [212].

In contrast to solid tumors of the periphery such as lung cancer, GBMs tend to harbor EGFR mutations affecting the ectodomain rather than the tyrosine kinase domain [213]. The most well-studied of these mutations, and the most common EGFR mutation found in GBM, is EGFRvIII [214]. An in-frame deletion of exons 2-7 results in the EGFRvIII mutant, which has a truncated extracellular ligand-binding domain, yet remains constitutively active despite its inability to bind to EGF or other growth factors [214, 215]. Additional EGFR mutations with truncated extracellular domains have been reported in glioma (deletion of amino acids 6-185, 521-603, and 118-164) [216, 217]. Due to the relative infrequency of these mutations in comparison to EGFRvIII, the prognostic value of these extracellular domain mutations has not been thoroughly investigated in response to all tyrosine kinase inhibitors, although one study found no clinical benefit to patients with EGFRvIII or other extracellular domain mutations in response to Dacomitinib, an irreversible EGFR inhibitor [218]. However, these extracellular domain variants also appear in combination with *EGFR* gene amplification and EGFRvIII [219], suggesting that *EGFR* amplification is the initial source of genetic instability in these tumors that gives rise to additional mutations within the protein [216].

Full-length EGFR is internalized after binding to EGF [220] as part of a feedback loop designed to regulate proliferation and survival transduction pathways. However, because EGFRvIII does not bind to any ligand as a result of its truncated extracellular domain, internalization signals are absent or significantly diminished, which results in constant, low-level activation of tumorigenic signals [221]. A meta-analysis of studies regarding the prognostic value of full-length EGFR in GBMs showed that high expression of EGFR was an indicator of shorter overall survival [222]. The impact of EGFRvIII on overall survival and progression-free survival is more controversial. Some studies determined that GBMs expressing EGFRvIII was a predictor of worse outcome in patients surviving greater than one year after diagnosis [223] and conferred treatment resistance to GBM cells [224], while additional groups found a neutral association between EGFRvIII expression and patient outcome [225]. Still, others found that EGFRvIII expression was correlated with *longer* overall survival and that these tumor cells were less resistant to TMZ than EGFRvIII negative GBM cells [226]. The lack of consensus within the literature regarding the prognostic value of EGFRvIII is likely due to variations in statistical methods, differing criteria and methods for determining EGFRvIII expression, pooling tissue from lower grade gliomas with GBMs, and the inherent genetic heterogeneity not only between patients, but also within the GBM of a single patient. Nevertheless, the presence of EGFRvIII can still be a promising therapeutic candidate as it is not expressed in noncancerous cells [227] and thus is currently under investigation in immunotherapy clinical trials to target GBM cells specifically [228, 229]. Although EGFR and EGFRvIII have shown promise as targets for GBM treatment, so far they have not proven to be the silver bullet needed.

1.6 EGFR, xCT, and CD44 in cancer: partners in crime or a coincidence?

It is clear that xCT, CD44 and EGFR all play important roles in the progression of multiple cancer types, possibly due to the convergence of signaling pathways that stimulate various pathways in GBM. Interestingly, CD44 and EGFR also appear to interact in diverse cell types that promote tumorigenic phenotypes. Fibroblasts modified to express wild type EGFR showed increased adhesion to a HA coated matrix in response to EGF, whereas very little adhesion occurred in cells that did not express wild type EGFR or were not exposed to EGF [230]. CD44S-HA binding was found to mediate the observed increase in adhesion, as incubation with CD44 blocking antibodies abrogated the adhesive ability of EGFR expressing cells, regardless of EGF treatment [230]. CD44 was also found to complex with EGFR in HSC-3 head and neck cancer cells [231]. Treating these cells with HA led to enhanced EGFR activity by increasing EGFR phosphorylation and the interaction between CD44 and EGFR [231]. A similar finding was demonstrated in oral mucosal fibroblasts, where cells were transfected with an expression vector for HAS2 to increase HA production, which led to increased CD44-EGFR co-localization on the cell membrane compared to control cells [232]. The converse was shown in C6 rat glioma cells, where inhibition of CD44-HA binding suppressed EGFR and AKT activation [233]. Moreover, CD44S expression on its own has been shown to induce transcription of the *HAS2* gene and resulted in increased downstream AKT signaling, and subsequently promoted cancer cell survival [166]. A functional relationship between CD44 and EGFR has also been observed in head and neck squamous cell carcinoma [234], and knockdown of CD44 expression resulted in reduced EGFR phosphorylation and downstream signaling in lung cancer [235, 236].

Because GBMs most commonly express CD44S, these cells may have evolved an elegant mechanism by which CD44S and EGFR are able to promote xCT-mediated glutamate release and

GSH production independent of CD44E expression. An additional clue as to how these relationships persist is provided by studies finding that depleted levels of CD44 increases EGFR degradation and sensitizes GBM cells to chemotherapy [237]. This could be a result of joint internalization of xCT and EGFR if the two proteins bind to each other, as has been shown in adherent GBM cells [143]. If this is the case, cells would lose access to the protective effects of increased intracellular GSH when xCT is internalized. Conversely, expression of CD44S inhibited the internalization and degradation of EGFR [237]. It is interesting to note that studies silencing CD44 expression or utilizing HYAL to degrade HA (both impairing the HA-CD44 interaction) show abrogated AKT signaling, which is a direct downstream target of EGFR [166]. This provides evidence that complex and nuanced interactions exist between xCT, EGFR, and CD44S in GBM and other cancers. These interactions appear to be contingent upon the type of cancer, the specific isoform(s) of CD44 and/or EGFR expressed, and the composition of the extracellular environment in which the cancer cell grows. Thus, the relationship between xCT, CD44-HA and EGFR has not fully been elucidated in the context of GBM, and warrants further study. These interactions are explored further in chapters 2 and 3.

1.7 Chapter summary and central hypothesis

GBM is a devastating disease that afflicts thousands of Americans every year. GBMs are highly invasive tumors that grow rapidly, resulting in patients surviving just around a year after diagnosis. As such, novel therapeutic approaches are desperately needed for the treatment of this deadly disease. Fortunately, there are still many avenues that have yet to be explored, and many target proteins expressed by GBM cells that are candidates for clinical intervention. Of particular interest are the xCT, CD44, and EGFR proteins that have previously been shown to have complex

roles in the pathogenic behavior of these tumors. The SXC transporter complex is upregulated in approximately 50% of GBM patients, contributing to increased glutamate release in peritumoral areas and increased GSH production inside the cell. While both EGFR and CD44 have demonstrated roles in GBM progression individually, they have also been shown to interact with xCT in GBM and gastrointestinal cancers, as well as with each other [146, 231, 232, 234]. However, little is known about how these interactions contribute to the biology and disease progression in GBM, especially regarding glutamate transport.

The primary goal of my thesis work was to determine if either CD44 or EGFR had a role in functional xCT regulation, and if so, whether this interaction could be exploited to decrease xCT-mediated glutamate release. Therapeutically, inhibiting xCT activity would reduce excess extracellular glutamate and concomitant tumor associated epilepsy, decrease GSH content within tumor cells, and potentially provide sensitization to treatment. **I hypothesize that the extracellular matrix molecules CD44 and EGFR independently regulate glutamate biology in GBM through functional xCT stabilization.** Chapters 2 and 3 describe the experimental undertakings that were devised to address this hypothesis, as well as the results and implications for CD44 and EGFR in future GBM therapeutic development.

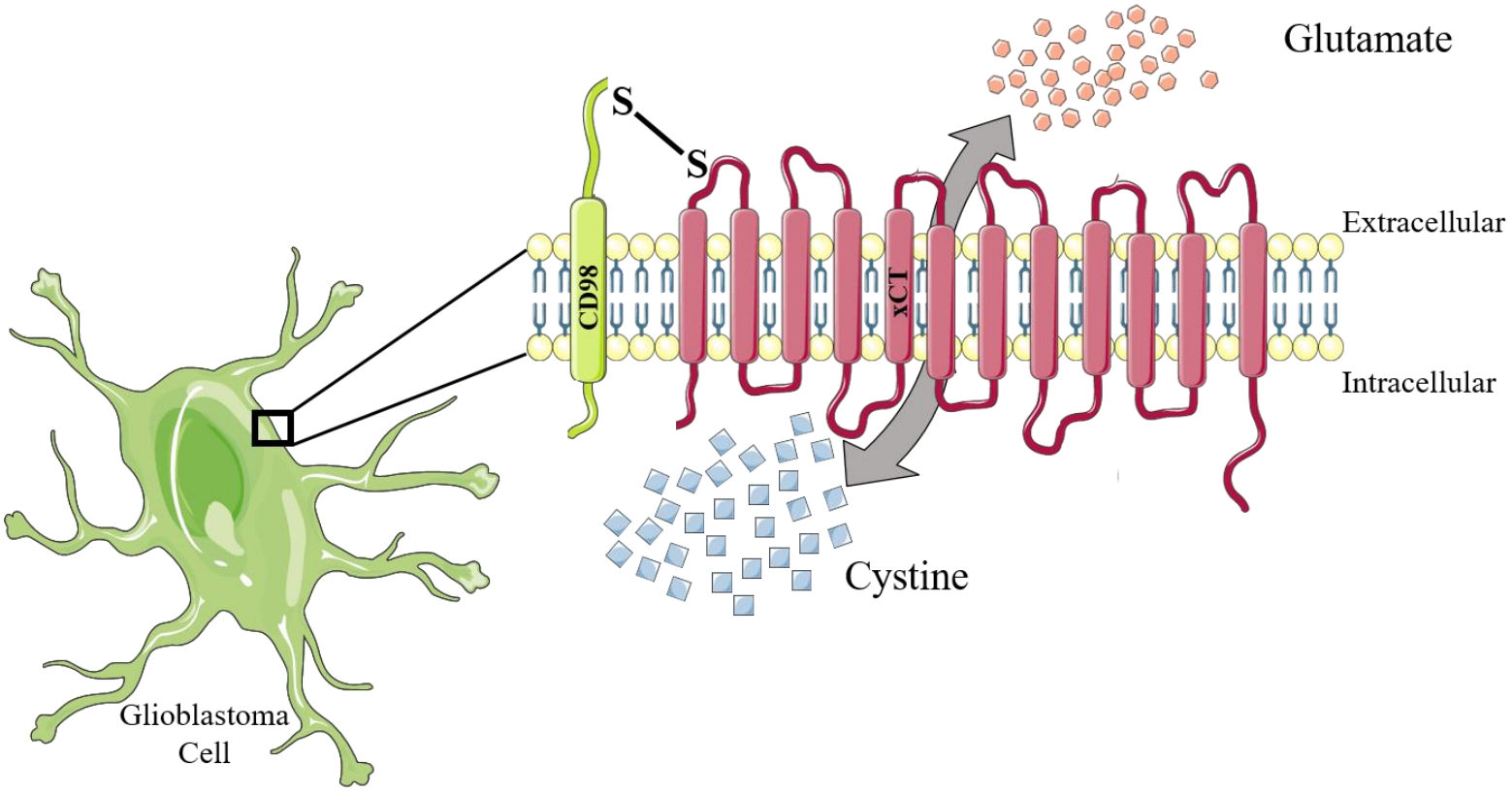

Figure 1.1. System x_c- on the plasma membrane of a glioma cell. CD98 (green) is the regulatory subunit of SXC, and is bound to xCT (pink), the catalytic subunit. SXC mediates the import of cystine (to be reduced into cysteine) for GSH synthesis, and simultaneously exports glutamate out of the cell. This image was created under Creative Commons License with Smart.Servier Medical Art.

A

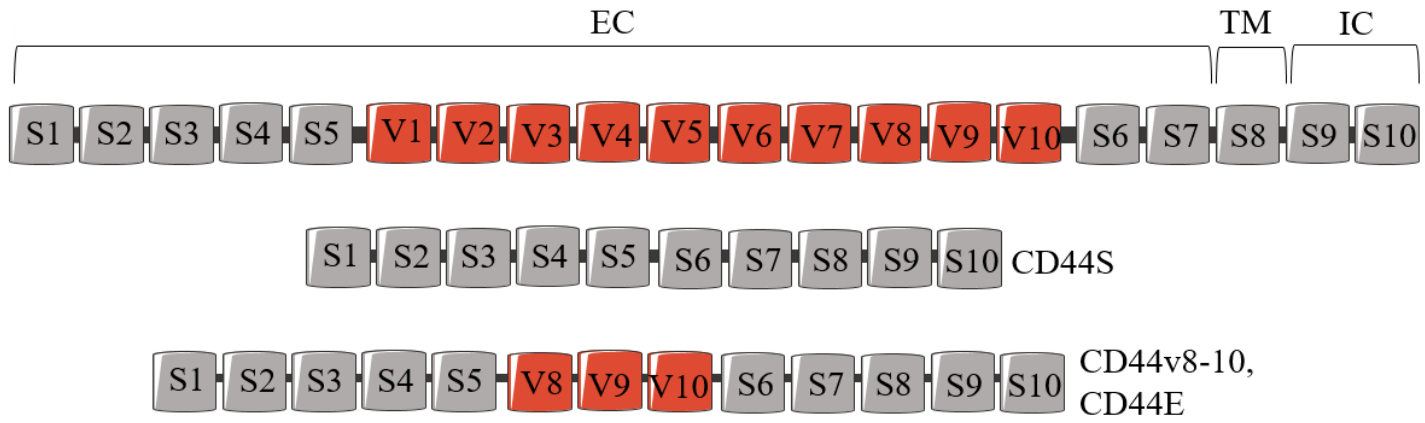

Figure 1.2 (A): Schematic of the CD44 gene. Alternative splicing results in many different CD44 isoforms, two of which are illustrated here. In humans, the standard isoform of CD44 (CD44S) contains all ten standard exons (S1-10, gray) and no variant exons (V-10, red). The epithelial isoform of CD44 (CD44E) contains all standard exons and variant exons 8-10. Exons S1-7 and V1-10 (if included) comprise the extracellular (EC) domain, exon S8 makes up the transmembrane (TM) domain, and exons S9-10 comprise the intracellular (IC) domain. This image was created under Creative Commons License with Smart.Servier Medical Art.

B

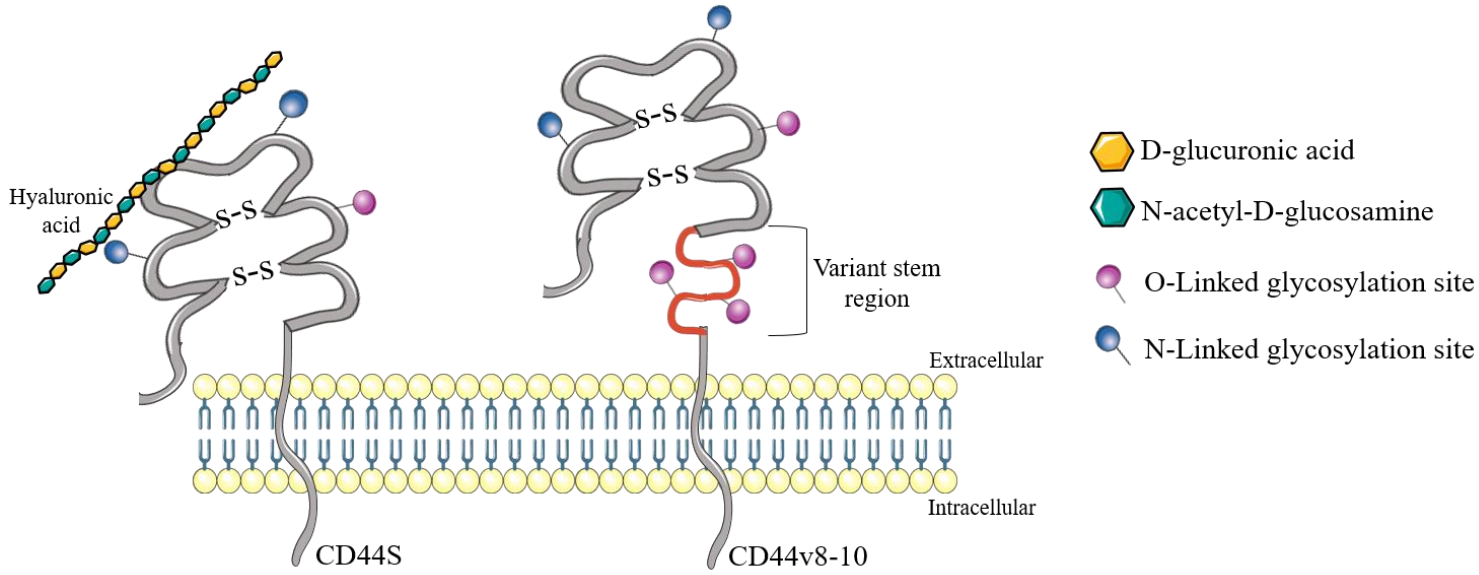

Figure 1.2 (B): Schematic of the CD44 protein. CD44 is a transmembrane protein and is best characterized as the principal receptor for hyaluronic acid. CD44S has the highest affinity for hyaluronic acid. CD44v8-10 has diminished hyaluronic acid binding capabilities due to increased O-linked glycosylation sites in the variant region. Amino acids coded by variant exons are inserted into the stem region of the protein (red). This image was created under Creative Commons License with Smart.Servier Medical Art.

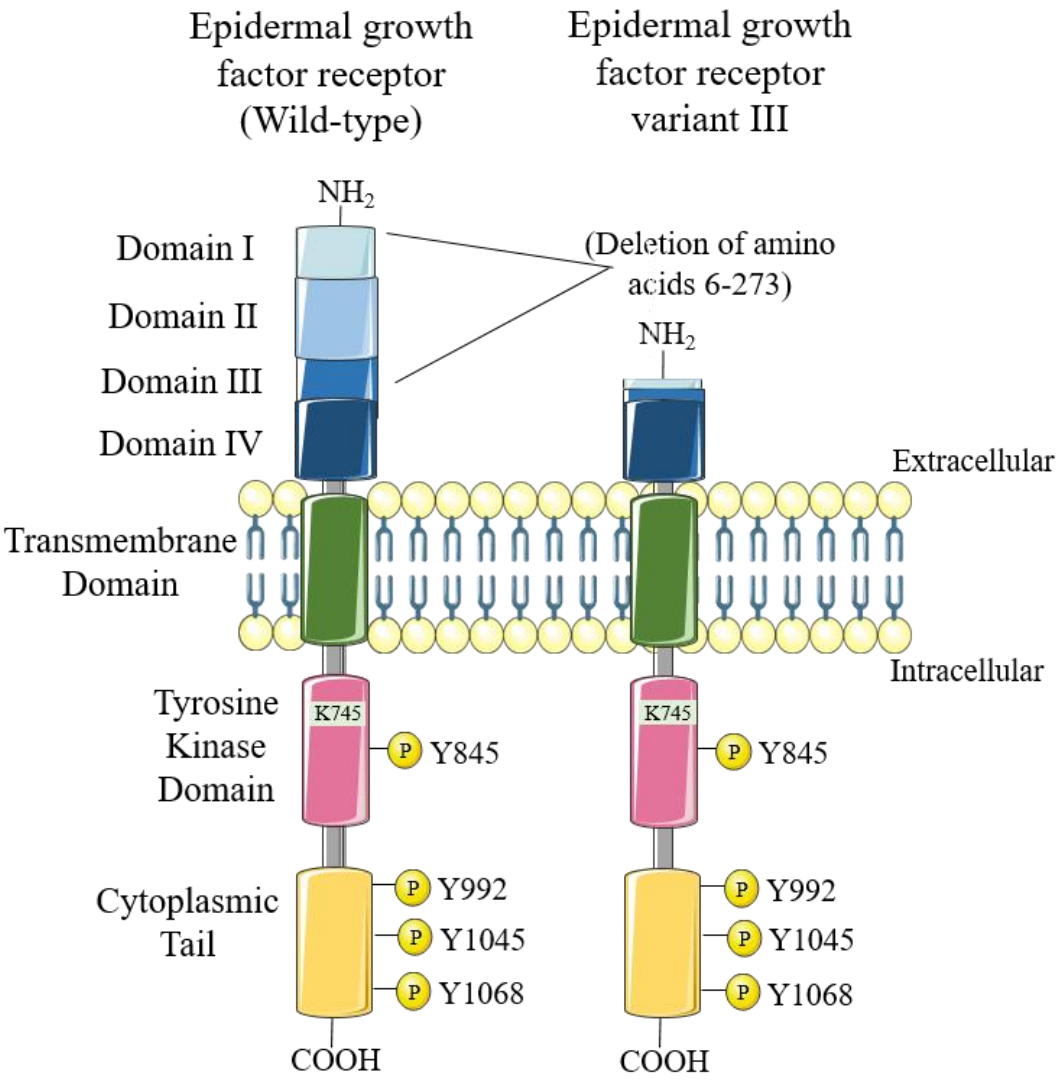

Figure 1.3: The epidermal growth factor receptor. EGFR is frequently amplified and mutated in GBM. EGFRvIII is the most common EGFR mutation found in GBM and has an in-frame deletion of exons 2-7 that affects the structure of the ligand binding domain. As such, this variant expresses constitutively active tyrosine kinase activity even in the absence of EGF. This image was created under Creative Commons License with Smart.Servier Medical Art.

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CHAPTER TWO: CD44-DEPENDENT REGULATION OF SYSTEM XC- IN GLIOBLASTOMA MULTIFORME

Joelle Martin and Harald Sontheimer

Abstract

Glioblastoma multiforme (GBM) is an advanced and aggressive brain cancer that develops from mutated glial progenitor cells. Treatment for GBM includes surgical resection, chemotherapy, and radiation, yet it remains an incurable disease with an average survival time of merely 12-15 months after diagnosis. Approximately 50% of GBM patients have tumors that overexpress a transmembrane protein known as system x_c- (SXC), which has been shown to promote rapid disease progression. SXC exports the neurotransmitter glutamate out of GBM cells in extremely high concentrations, thereby creating an excitotoxic environment for surrounding neurons, while simultaneously facilitating tumor invasion. Another protein implicated in promoting GBM invasion is CD44. HA is the primary binding ligand for CD44, and both molecules have demonstrated roles in promoting GBM cell survival and migration. Additionally, CD44 has been shown to regulate SXC in gastrointestinal and head and neck cancer. To determine if this regulatory relationship is conserved in GBM, we used two patient-derived GBM cells for experimentation. We found that GBM cells produced endogenous HA, and that HA expression was positively correlated with SXC expression. However, futher experimentation with pharmacological inhibition of HA and knockdown of CD44 with siRNA provided no evidence that SXC is regulated by CD44 or HA in GBM cells.

Introduction

Glioblastoma Multiforme (GBM) is the most malignant form of adult brain cancer, with 95% of patients succumbing to their disease within 5 years of diagnosis [1]. One factor contributing

to this poor prognosis is upregulation of the transporter system x_c- (SXC). SXC is a membrane bound antiporter that mediates the equimolar exchange of intracellular L-glutamate for extracellular L-cystine [2]. On average, GBM patients with high SXC expression have shorter survival times and a higher incidence of tumor-associated epilepsy compared to GBM patients with low SXC expression [3]. SXC leads to a worse prognosis by exporting excitotoxic concentrations of glutamate into the extracellular space, where peritumoral neurons become hyperexcitable and eventually die [4]. An additional consequence of upregulated xCT activity is the obligatory import of cystine into the cell to be utilized as a precursor for glutathione (GSH) synthesis [2]. GSH is an important antioxidant for cells, and increased concentrations of internal GSH protects tumor cells from alkylating chemotherapeutics such as temozolomide (TMZ), rendering GBMs particularly recalcitrant to treatment [5].

Multiple *in vivo* studies have demonstrated that inhibiting xCT activity with the drugs sulfasalazine (SAS) or (S)-4-carboxyphenylglycine prolonged survival while also decreasing seizure incidence and peritumoral edema in mice, indicating that xCT inhibition is a promising target for GBM therapy development [6-9]. However, phase I/II clinical trials involving SAS as a treatment for GBM patients ultimately failed due to a lack of efficacy in decreasing tumor burden (likely due to a short biologically active half-life) [10], as well as study participants experiencing adverse events [11, 12]. Because pharmacological inhibition of xCT has been met with limited success in humans, molecular regulators of xCT in GBM are currently being explored with increased enthusiasm – one particular candidate is the signaling protein CD44.

CD44 and its principal binding ligand hyaluronic acid (HA) have both been implicated in promoting GBM progression, migration, and invasion [13-15]. CD44 is a well studied transmembrane signaling molecule that undergoes extensive alternative splicing, resulting in many

different isoforms of CD44 throughout the body [16]. Variant isoforms of CD44 (CD44v8-10, CD44v9) have been shown to interact with xCT in multiple cancers, including gastrointestinal cancer, lung cancer, and other epithelial carcinomas [17-20].

CD44 and HA work in tandem to support tumor growth and viability- a partnership that is clearly demonstrated in the process of gliomagenesis. CD44 expression is positively correlated with brain tumor severity and was more focally expressed in the tumor core relative to the tumor margin or periphery [21, 22]. High CD44 expression in GBMs is associated with overall poor prognosis, and a study of human patients discovered that patients with lower levels of CD44 survived longer than those with high CD44 expression (median survival 18.5 months vs 3.5 months, respectively) [23]. CD44S appears to be the dominant form of CD44 found across all brain tumor types, with additional variants observed in GBM as well as lower grade gliomas [24, 25].

Of note in GBM is the absence of the variant CD44E (CD44v8-10), which contains variable exons 8-10 and is primarily found in epithelial tumors [17, 26]. This is interesting because CD44E has compromised HA binding due to extensive O-linked glycosylation [27]. The dominance of CD44S, which has the greatest capacity for HA binding of all the variants, and lack of CD44E, which has minimal HA binding capability, in GBM suggests an important role for the CD44-HA interaction in GBM cell survival and migration. Indeed, the concentration of HA is higher surrounding glial tumors than in control cortex [28]. CD44 variants have been implicated in the metastatic process of multiple types of solid tumors [29], and the abundance of CD44S relative to CD44v in GBM may at least partially [30] explain why these tumors so rarely metastasize out of the brain, yet are highly invasive within the brain [26]. *In vitro* studies showed that GBM cell invasion was significantly abrogated in modified Boyden chamber assays and traditional transwell

invasion assays after the CD44-HA interaction was inhibited by CD44-neutralizing antibodies, genetic knockdown of CD44 expression, or removal of HA from the system [31-33].

A study in HCT116 gastrointestinal cells showed that CD44 (specifically CD44E) is a binding and stabilization partner for xCT, and that this interaction confers additional defense against ROS to the cells [18]. This relationship also exists in lung cells afflicted by pulmonary hypertension, revealing a preference of xCT to bind with CD44E rather than CD44S, and that the v8-10 region of the CD44E protein was necessary for this interaction [18, 34]. The CD44E-xCT complex also enhanced GSH production and the metastatic potential of breast cancer cells into the lung [35]. However, this relationship does not appear to be present in GBM [17], likely due to the fact that GBM cells rely so heavily on the CD44-HA signaling axis for tumor cell survival and propagation.

HA plays a role in regulating extracellular glutamate concentrations by assisting in the localization and activity of the glutamate transporters GLT1 and GLAST in the membrane of astrocytes *in vitro.* Proper glutamate transporter trafficking is inhibited by the addition of HYAL, which leads to excitotoxic levels of glutamate in this paradigm [36]. The ability of HA to orchestrate glutamate transporter localization occurred independently of the CD44-HA interaction, but this data nevertheless suggests a role for HA in regulating glutamate release via glutamate transporters.

Our study utilized two GBM patient-derived xenograft (PDX) lines that express low levels of xCT (GBM14) and high levels of xCT (GBM22). PDX cells are powerful models used to identify candidate drug targets due to enhanced preservation of gene expression over traditional adherent cell lines [37, 38]. The goal of our study was to determine if CD44 and/or HA played a role in the

regulation of xCT in GBM PDX cells. The results of our experiments confirmed that neither HA or CD44 appear to have a role in regulating xCT expression or function in either PDX cell line.

Methods and materials

Cell culture

GBM patient-derived xenograft (PDX) cells (GBM12, GBM14, and GBM22) were a generous gift from Dr. Yancey Gillespie and Catherine P. Langford (Brain Tumor Tissue Core, University of Alabama at Birmingham, Birmingham, AL, USA). PDX cells were maintained through serial passage in athymic nude mice as previously described [39]. Briefly, tumor tissue was harvested 14-21 days post-injection and the tissue was harvested, homogenized, and approximately 200µl of tissue was injected subcutaneously into the flank of athymic nude mice for tumor propagation. The remaining tissue was suspended in RPMI 1600 media supplemented with enzymes A, R and K (Tumor Dissociation Kit, Miltenyi Biotec, Cat# 130-096-730), and later dissociated mechanically with a GentleMACS tumor Dissociation Kit. Cells were passed through a sterile 40µm filter and GBM14 and GBM22 cells were maintained as tumor spheres in DMEM/F12 medium (Thermo Fisher, Cat# 21-041-025) supplemented with 10 ng/ml FGF (Thermo Fisher, Cat# PHG0261), 10 ng/ml EGF (Thermo Fisher, Cat# PHG0311), 2% B-27 Supplement w/o Vitamin A (Thermo Fisher, Cat# 12587010), 250 µg/ml amphotericin (Fisher, Cat# BP264550), and 50mg/ml gentamycin (Thermo Fisher, Cat# 15750060), and incubated in 10% CO_2 at 37°C. GBM12 cells were maintained in Neurobasal medium (minus phenol red, ThermoFisher/Gibco, Cat# 12348017). Neurobasal medium is prepared the same way as DMEM/F12 media, with the exception of adding 260mM L-glutamine (ThermoFisher, Cat # 25030081), and cells were maintained in 10% CO_2 at 37°C. D54 and U251 glioblastoma cells were gifts from Dr. D. Bigner (Duke University, Durham, NC, USA), and Dr. G. Yancey Gillespie (University of Alabama at

Birmingham, Birmingham, AL, USA), respectively. Cells were grown in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 medium (DMEM/F-12) supplemented with 7% (D54) or 10% (U251) fetal bovine serum (FBS; Aleken Biologicals, Texarkana, TX, USA) at 37°C and 10% CO_2. HCT116 cells were purchased from the American Type Culture Collection and maintained in DMEM/F-12 medium supplemented with 10% FBS at 37°C and 5% CO_2. All cells used for glutamate or GSH assays were incubated in Neurobasal medium. Primary GBM tissue used was provided by the neurosurgery team at Carilion Regional Memorial Hospital under IRB #15-670. Human astrocyte cDNA was a generous gift from Dr. Jennifer Munson (Fralin Biomdical Research Center, Roanoke, VA). All cell lines were tested and free of mycoplasma contamination (VWR, Cat# 10497-508).

Cell culture treatment

Hyaluronidase treatment *in vitro:* PDX cells were incubated for the indicated times with 0.5 mg/ml of hyaluronidase from bovine testes (Sigma, Cat# H3884-100MG) solubilized in Neurobasal medium. Hyaluronic acid treatment *in vitro:* PDX cells were incubated for the indicated times with 0.5 mg/ml hyaluronic acid sodium salt from *Streptococcus equi* (Sigma, Cat# 53747-1G) solubilized in Neurobasal culture medium.

Western Blot

Western blotting was performed as previously described [3] with modifications. PDX cells were lysed in RIPA buffer (final concentration 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris Base, pH 8.0), supplemented at 1:100 with protease (Sigma, P8340) and phosphatase (Sigma, P0044) inhibitor cocktails. Protein concentrations were normalized across samples and 15-45 µg of sample lysate was run on a 4-20% gradient precast SDS-polyacrylamide gel (Bio-Rad Cat# 4561094), then transferred (semi-dry) onto a polyvinylidene difluoride (PVDF) membrane (ThermoFisher Cat# 88518) using a BioRad Trans-

blot Turbo Transfer System at 1.3 amps and 25 volts for 10 minutes. Membranes were blocked in 5% nonfat dry milk in TBS-T (0.1% Tween-20 in Tris-buffered saline, pH 7.4) for 1 hour at room temperature with gentle rocking. Membranes were incubated with primary antibodies [goat anti-xct (1:450; Abcam; ab60171), rabbit anti-CD44 (1:250; Sigma, HPA005785), chicken anti-glyceraldehyde-3-phosphate dehydrogenase (1:5000; Millipore Sigma; AB2302), rabbit anti-Na^+/K^+ ATPase (5µg:3mL; Abcam; ab76020), rat anti-alpha-tubulin (1:500; BioRad; MCA77G)] in blocking buffer overnight at 4°C, then washed 4x10 minutes in TBS-T. Membranes were incubated with HRP-conjugated secondary antibodies: [rabbit anti-goat (1:1500; Santa Cruz; 2922), goat anti-rabbit (1:5000; Invitrogen; 65-6120), donkey anti-rat 568 (1:1000; Biotium-20092), donkey anti rabbit 647 (1:1000; Jackson Immunoresearch; 711-605-152), goat anti-chicken (1:2000; Santa Cruz-2901)]. Blots using HRP-conjugated secondary antibodies were developed using Supersignal West Femto (Thermo Fisher, Cat# 34095) and imaged on an Azure Biosystems 500 imager. Fluorescent secondary antibodies were used for the Mem-Per Kit western blots only and visualized on a BioRad ChemiDoc MP imaging system. Band intensity values were generated using FIJI software, and experimental protein values were normalized to GAPDH. Images of western blots are representative of 3 independent replicates.

RNA isolation, RT-PCR, and Quantitative RT-PCR

RNA isolation: Cells were plated at 1 million cells in 2ml of either control or experimental DMEM/F12 media. RNA was isolated from the cell samples using the Purelink RNA Mini Kit (Thermo Fisher, Cat# 12183025). cDNA was then synthesized (stock concentration 50 ng/µL) using SuperScript VILO Master Mix (ThermoFisher, Cat# 11755500) at 25˚C for 10 minutes, 42˚C for 1 hour, 85˚C for 5 minutes, then stored at -20˚C.

RT-PCR: Expression of CD44S, CD44E, and GAPDH were determined by loading cDNA to each lane and running gel electrophoresis. Primer pairs were as follows: CD44S, Forward: 5'-CCGCTATGTCCAGAAAGGAG-3' and Reverse: 3'-GGTCTCTGGTAGCAGGGATTCTG-5'. CD44E, Forward: 5'-TCCCAGACGAAGACAGTCCCTGGAT-3' and Reverse: 3'-CACTGGGGTGGAATGTGTCTTGGTC-5'. GAPDH, Forward: 5'-ACCACAGTCCATGCCATCAC-3' and Reverse: 3'-TCCACCACCCTGTTGCTGTA-5'. PCR reactions were optimized to 95ºC for 2 minutes, 35 amplification cycles at 95ºC, the appropriate annealing temperature (54ºC for CD44S and GAPDH, 60ºC for CD44E) for 30 seconds, 72ºC for 30 seconds, and a final extension at 72ºC for 5 minutes. Amplified products were run on 2% agarose gels and visualized with GelRed Nucleic Acid Stain (Biotium Inc, Cat #41003-T).

qRT-PCR: Expression of *SLC7A11, CD44,* and *GAPDH* was determined using quantitative real-time PCR. Each cDNA sample (100 ng) was amplified using Taqman reagents on an ABI StepOnePlus real-time PCR System. Taqman probes from ThermoFisher were as follows: *SLC7A11:* Hs00921938_m1, *CD44*: Hs01075862_m1, and *GAPDH:* Hs02758991_g1. The ΔCt method was used for baseline mRNA expression quantification. *CD44* and *SLC7A11* values were made relative to *GAPDH*. The ΔΔCt method was used to determine relative differences in *SLC7A11* mRNA between control cells and those treated with HYAL. Data from three technical replicates were confirmed to be under a standard deviation of 25%. Transcript expression levels were confirmed by three independent biological replicates, and each sample was run with three technical replicates.

Membrane Permeabilization Kit

The Mem-PER™ Plus Membrane Protein Extraction Kit (Thermo Fisher, Cat# 89842) was utilized to separate membrane-bound protein fractions from cytosolic protein fractions. The experiment was conducted according to manufacturer's instructions for suspension mammalian cells, with

some variations. Five million cells were seeded into 15 mL of control media or media supplemented with 0.5mg/mL hyaluronidase and left to incubate for 24 hours. Permeabilization and solubilization buffers were supplemented with protease and phosphatase inhibitors (1:100, see western blot). After step 4, the supernatant was aspirated with a U-100 insulin syringe and an additional 5 minute wash step in 500 µL of cell wash buffer was added. Once the final fractions were established, protein concentration was normalized (Pierce BCA Protein Assay Kit, ThermoFisher, Cat# 23225) and membranes were probed for xCT, Na^+/K^+ ATPase, and α-tubulin.

Colorimetric Glutamate Assay

PDX cells were dissociated with Accutase Solution (Sigma, Cat# A6964) and seeded at 250,000 cells per 0.5 mL of control or experimental media. Cell media was collected after 24 hours, and glutamate concentrations were measured using a glutamate assay kit (Sigma-Aldrich, Cat# MAK004) according to the manufacturer's instructions. The experiment was set up in technical triplicates and each was run in duplicate on the assay plate. Due to the difference in glutamate concentrations from the two cell lines, 50 µL of sample from GBM14 cells and 5 µL of sample from GBM22 cells was used. The difference in sample volumes was accounted for by using the equation provided in the instruction manual. HYAL treated samples (and controls for these experiments) were processed through 10K centrifugal filter devices (Fisher, Cat# MRCPRT010) for 40 minutes at 14,000xg to remove HYAL, which interferes with glutamate readings. Cells were collected in 60 µL RIPA buffer (see western blot) and total protein concentrations for each well were matched to glutamate concentrations to determine glutamate:protein ratios. Data for these experiments is representative of three biological replicates.

Immunocytochemistry

PDX cells were dissociated and plated on glass coverslips pre-coated for at least 4 hours with poly-L-ornithine (Poly-O) solution (Sigma, Cat# P4957) diluted 1:100 in DMEM/F12 media. 200K cells were allowed to adhere to the coverslip overnight. The next day, fresh media was added and cells were left for 24 hours. The coverslips were fixed with a 4% PFA solution in PBS for 15 minutes at room temperature (RT), then washed with cold PBS for 3x5 minutes. Coverslips were blocked (PBS with 10% donkey serum and 0.1% Tween-20) for 30 minutes at RT. Primary antibody: hyaluronic acid binding protein (Millipore Sigma Cat# 385911) was diluted 10 µg/ml in blocking solution, and incubated on the coverslip for one hour at RT. Coverslips were washed 6x10 minutes with PBSTw (PBS with 0.1% Tween-20). Secondary antibody: Streptavidin 647 (Jackson Laboratories Cat# 016-600-084) was diluted 1:500 in blocking solution and incubated on the coverslip for one hour at RT, protected from light. Coverslips were washed 6x10 minutes with PBSTw. DAPI (Thermo Fisher, Cat# D1306) was diluted in blocking solution at 1:1000 and incubated at RT for 5 minutes. The coverslips were mounted with Fluoromount G Mounting Solution (Electron Microscopy Sciences, Cat# 17984-25). Images were acquired using an A1R Nikon Laser Scanning microscope with a Plan Apo 20X/N.A.1.40 air objective. Data for these experiments is representative of three biological replicates, each with 5 technical replicates.

Short Interfering RNA (siRNA)

CD44 siRNA, SMARTpool: ON-TARGETplus CD44 siRNA, 5 nmol (Dharmaon, Cat # L-009999-00-0005) was used to knockdown CD44 expression. 5X siRNA buffer (Dharmacon, Cat# B-002000-UB-100) was diluted to 1X in RNase-free water and used to reconstitute siRNA to 10 µM. Cells were plated at 250,000 per well and transfected using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher, Cat# 13778030), Opti-MEM I Reduced Serum Medium (Thermo Fisher, Cat# 331985062) and antibiotic-free DMEM for 24 hours. Cells were then

switched to complete DMEM, and 24 hours before the conclusion of the experiment, cell media was switched to Neurobasal (if cell media was to be analyzed for extracellular glutamate concentration). The total time for CD44 siRNA transfection was 72 hours. ON-TARGETplus Non-targeting Control Pool, 5 nmol (GE Healthcare Dharmacon, Cat# D-001810-10-05) was used as a control and reconstituted in the same manner as experimental siRNAs. The final concentration of siRNA was 100nm siRNA per well. All siRNA experiments utilized three biological replicates.

Hyaluronic acid quantification

Hyaluronic acid concentration was quantified using the Hyaluronan Quantikine ELISA Kit (R&D Systems/Fisher, Cat# DHYAL0) according to manufacturer's instructions. Briefly, cells were plated in Neurobasal media (250K/500µL) for 24 hours. Media was separated from the cells via centrifugation and diluted 1:80 in the provided calibration diluent. Samples were then analyzed according to manufacturer's instructions. HA concentration values were then made relative to the respective protein concentration value from the same well. All data is representative of three biological replicates, each including three technical replicates.

Intracellular Glutathione Measurements

Intracellular GSH was quantified using the QuantiChrome™ glutathione assay kit (BioAssay Systems DIGT-250). Cells were plated (250K/500µL) in Neurobasal media, and experimental samples were incubated with .5mg/mL of HYAL supplemented Neurobasal media for 24 hours. Cells were then processed according to the manufacturer's instructions for cell lysate preparations. The plate was read after a 10 minute incubation at room temperature. Data is representative of 3 biological replicates, each with 3 technical replicates.

Statistical Analysis

Results are presented as means ± SEM. Significance was defined as $*p < 0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$. GraphPad Prism 8.0.1 was used for statistical analysis. Normally distributed data comparing two groups were subjected to two-tailed Student's *t* tests.

Results

xCT and CD44 expression in GBM xenograft cells

Previous work from our lab reported that approximately half of patient-derived gliomas highly express the *SLC7A11* gene encoding for the catalytic subunit xCT, while the remaining gliomas have low to almost undetectable expression of *SLC7A11* [3]. We selected two examples of GBM cells (GBM14 and GBM22) which have low (GBM14) and high (GBM22) expression of *SLC7A11* (Figure 2.1A). Gene expression correlated perfectly with protein levels (Figure 2.1B), and the high *SLC7A11* transcript and xCT protein expression resulted in predictably higher extracellular glutamate concentrations in GBM22 cells compared to GBM14 cells (Figure 2.1C). CD44 is reported to stabilize xCT in multiple cancers and promote glioma pathogenesis [13, 18, 20]. Analysis of *CD44* transcript was significantly higher in GBM14 cells compared to GBM22 cells (Figure 2.2A). However, this difference was not seen at the protein level (Figure 2.2B). This discrepancy is likely due to the presence of additional CD44 variant isoforms in GBM14 cells that are not present in GBM22 cells or a higher concentration of non-coding mRNAs produced by GBM14 cells.

GBM cells with high xCT expression have increased hyaluronic acid production

HA has been shown to play an important role in supporting GBM invasion [32] and regulating glutamate transporter localization [36], which led us to consider the CD44-HA interaction as a candidate xCT regulator. To determine the extent to which our GBM cells synthesized HA, and if

there was a relationship between HA concentration and xCT expression, we measured endogenous levels of HA in the media of our GBM cells. GBM22 cells produced significantly more extracellular HA than GBM14 cells (Figure 2.3A). Additional immunofluorescence experiments supported this finding, and we quantified significantly higher hyaluronic acid binding protein (HABP) signal from GBM22 cells in comparison to GBM14 cells (Figure 2.3B). Together, these data indicate there is a positive relationship between xCT expression and HA production in GBM14 and GBM22 cells. Next, we examined if adding HA to cell media would increase glutamate release. This was tested by first treating cells with 0.5mg/mL HYAL for 24 hours to degrade any HA that was currently bound to receptors. Cells were then incubated for an additional 24 hours in either control media or media supplemented with 0.5mg/mL HA. We observed no difference in glutamate release at this second time point, indicating that at 24 hours, our PDX cells reach a ceiling of HA-receptor engagement (Figure 2.4A,B). Because our cells readily produce HA, we determined that degrading HA with HYAL, rather than adding additional HA, would be the best way to investigate the role of the CD44-HA interaction in regulating xCT.

Enzymatic degradation of HA does not affect xCT expression or xCT-mediated glutamate release

Hyaluronidase (HYAL) is a naturally occurring enzyme that breaks down HA [40]. To determine if elimination of HA would reduce xCT expression and extracellular glutamate concentration, we incubated our PDX cells with control media or media supplemented with HYAL for 24 hours. Analysis of *SLC7A11* transcript revealed no significant difference between control and HYAL conditions in either cell line, indicating that HYAL does not have the ability to regulate *SLC7A11* transcription (Figure 2.5A,B). Using the same experimental paradigm, GBM14 and GBM22 cell lysates were analyzed via western blot to determine xCT protein expression. Semi-quantitative western blot analysis revealed no change in xCT expression after HYAL incubation, indicating

that HYAL does not affect post-transcriptional regulation of xCT in either cell line (Figure 2.6A,B). Additionally, we isolated membrane proteins to determine if HYAL treatment had an effect on xCT localization. These experiments also indicated no change in xCT expression on membranes due to HYAL incubation, indicating that the elimination of HA does not affect xCT protein localization (Figure 2.7A,B). We next sought to determine if reducing the availability of HA to our PDX cells would affect xCT activity. We measured extracellular glutamate from PDX cells treated with control media or media supplemented with HYAL after a 24 hour incubation. No difference in glutamate was observed in either cell line as a result of HYAL incubation, indicating that HYAL does not affect xCT function (Figure 2.8A,B). As another method of evaluating xCT function, we analyzed intracellular GSH levels in PDX cells under the same conditions. Intracellular GSH concentrations remained the same between control and HYAL-treated conditions in both cell lines (Figure 2.9A,B). However, GSH levels are significantly higher in GBM22 cells compared to GBM14 cells, as expected given the differential expression of xCT in the two cell lines (Figure 2.9C). Taken together, our data demonstrate that the presence of HA is not necessary for xCT expression, localization, or function.

CD44 does not regulate xCT in GBM cells expressing CD44S

Next, we explored the relative importance of CD44 on xCT-mediated glutamate release. We utilized siRNA to knockdown CD44 expression, and measured glutamate release 72 hours post-transfection from our PDX cells. Again, we observed no difference in extracellular glutamate concentration in the media of cells treated with CD44 siRNA or non-targeting siRNA (Figure 2.10A,B). Previous studies have implicated a particular variant of CD44 (CD44v8-10, CD44E) as a regulator of xCT expression [18]. Finally, transcript expression of the standard form of CD44 (CD44S), which contains no variable exons, and CD44E was examined in GBM cell lines, a

gastrointestinal cancer cell line (HCT116), and human astrocytes. Interestingly, none of our GBM cells or astrocytes expressed CD44E, but all cells expressed CD44S. This is in agreement with previous literature [18] suggesting that additional amino acids coded by variant exons present in the isoform CD44E are necessary for binding to xCT, and that CD44S lacks this binding capability (Figure 2.11).

Discussion

GBMs are notorious for infiltrating into deep recesses within the brain as well as establishing satellite tumors after migrating along brain vasculature and white matter tracts [41]. While important, individual mutations are only partially responsible for the propensity of GBM cells to migrate and invade. The complete study of these mutations and mechanisms must include the environmental context in which GBM tumors grow. Hyaluronic acid is a major component of the extracellular space of most tissues and is instrumental in signal transduction pathways that promote cell adhesion, migration, invasion, and tissue remodeling [15]. Hyaluronic acid is the principal glycosaminoglycan found in the extracellular matrix and tumor microenvironment, as it is found in higher concentrations near GBM tumor stroma compared to surrounding healthy tissue [15, 28]. Additionally, the presence of HA has been shown to contribute to GBM invasion alone and through binding with CD44 [31, 42].

Specifically, the variant CD44E has been implicated as a stabilizing partner for xCT in gastrointestinal cancer by binding to xCT through its variable stem loop region [18]. However, this variant is uncommon in GBM, an unsurprising finding considering that CD44E has limited HA binding capacity due to O-linked glycosylation preventing HA from docking efficiently [17, 27]. Thus, the standard form of CD44, which has the greatest capacity for HA binding, is also the most abundant form of CD44 found in normal brain as well as in GBM tissue [25, 43]. Although

HA has a simple structure of repeating units of N-acetyl-D-glucosamine and D-glucuronic acid, it performs complex functions. High molecular weight HA signaling has immunosuppressive and structure supporting functions, while low molecular weight HA signaling leads to pro-inflammatory, pro-migratory, and angiogenic effects [44]. In GBM, matrix metalloproteinases and hyaluronidases work in tandem throughout peritumoral regions to break down HA and promote tumor cell migration [14, 45]. Additionally, a recent study showed that HA is necessary for excitatory amino acid transporters GLT1 and GLAST recruitment to astrocytic processes and extracellular glutamate clearance [36]. These adjacent findings speak to the vast effects HA signaling can have in different tissues and disease states, and demonstrates that many complexities of HA remain to be discovered.

Conclusions

In summary, we found a correlation between xCT expression and HA production in our PDX lines. However, this did not translate into a biologically meaningful relationship between xCT and CD44/HA in GBM cells. By eliminating HA through enzymatic degradation, we evaluated the transcript expression, total protein expression, and membrane expression of xCT, as well as glutamate release, but did not find evidence to support that xCT expression or function is related to the CD44-HA interaction in our PDX cells.

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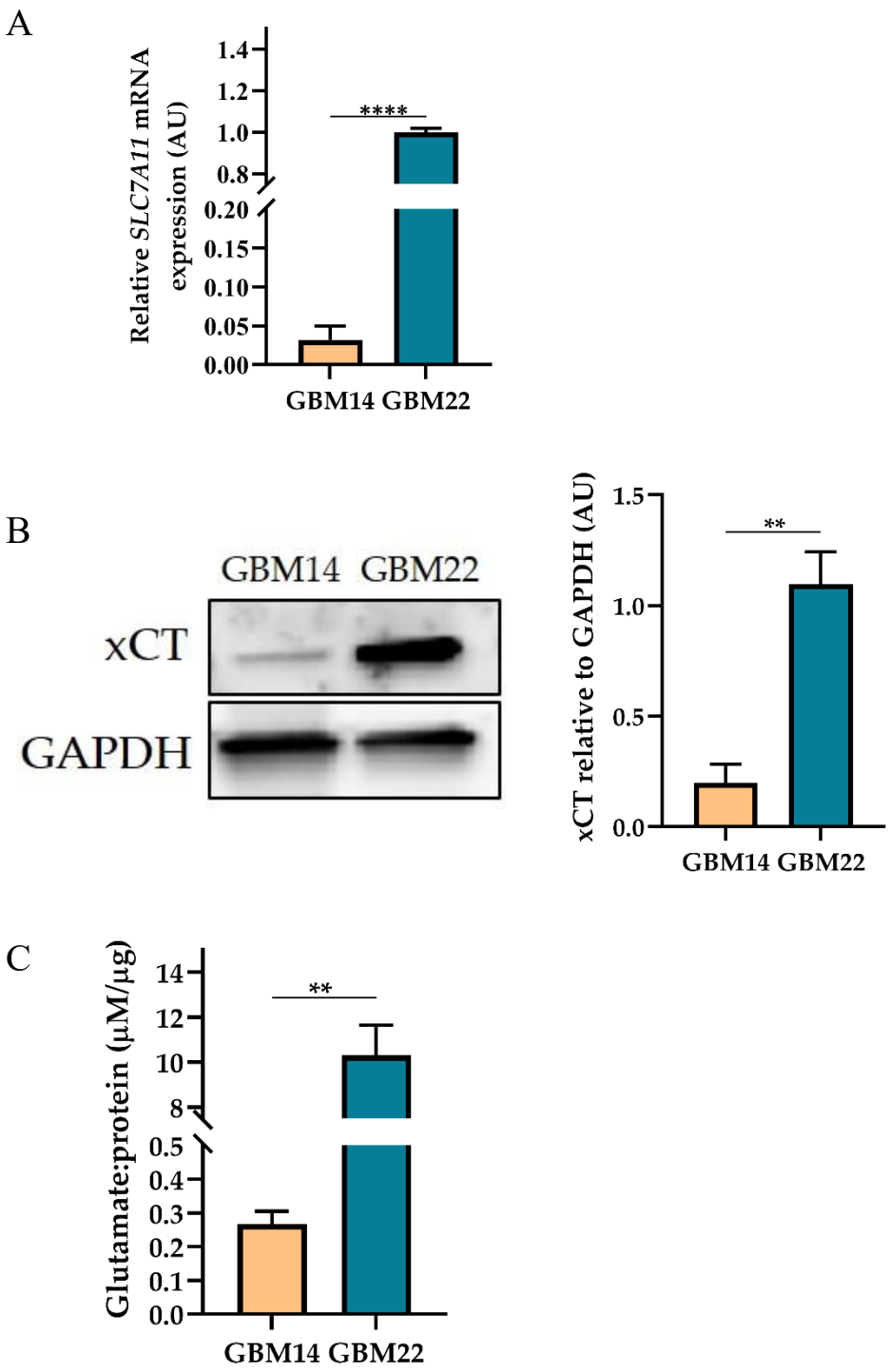


Figure 2.1: Baseline expression and function of xCT in two GBM patient-derived xenograft (PDX) cell lines. (A) *SLC7A11* transcript expression relative to GAPDH, N=3. (B) Representative western blot of xCT expression in PDX cells relative to GAPDH and quantification of xCT band intensity relative to GAPDH, N=3. (C) Baseline levels of extracellular glutamate concentrations in PDX cells, N=3.

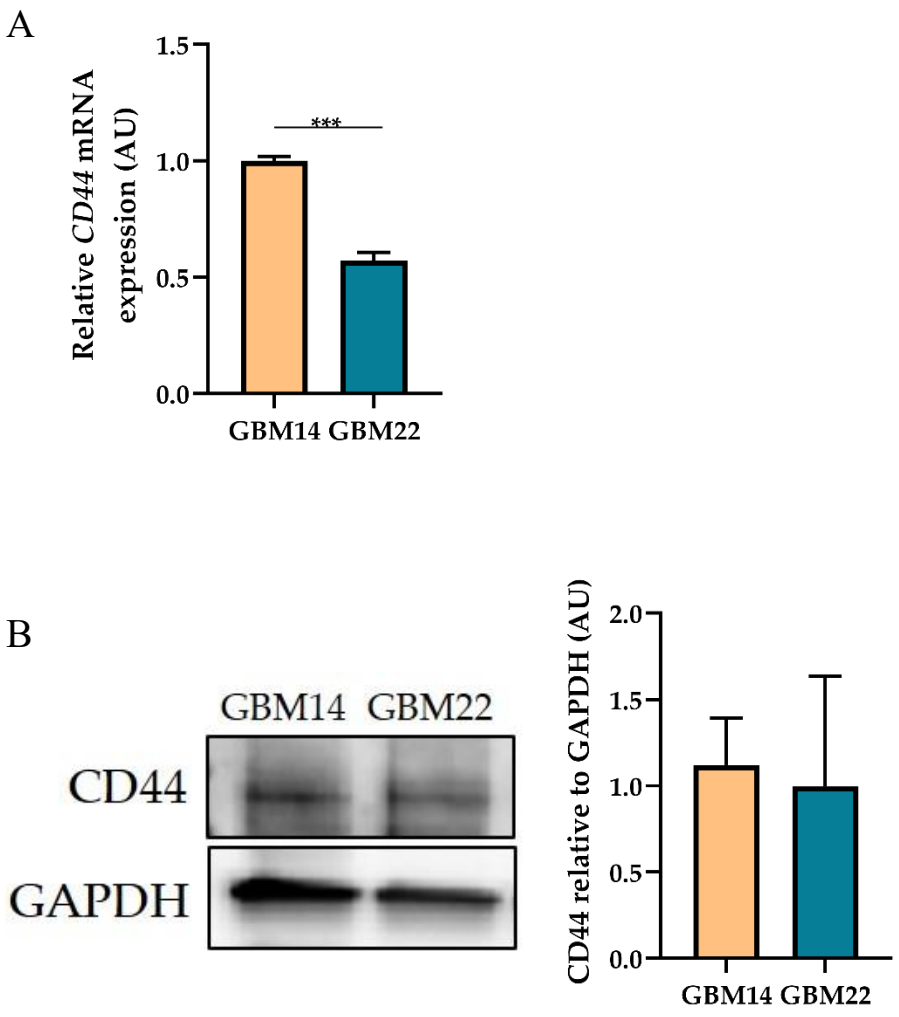


Figure 2.2: CD44 expression in GBM14 and GBM22 cells. (A) CD44 transcript expression relative to GAPDH, N=3. (B) Representative western blot of CD44 expression in PDX cells, and quantification of CD44 band intensity relative to GAPDH, N=3.

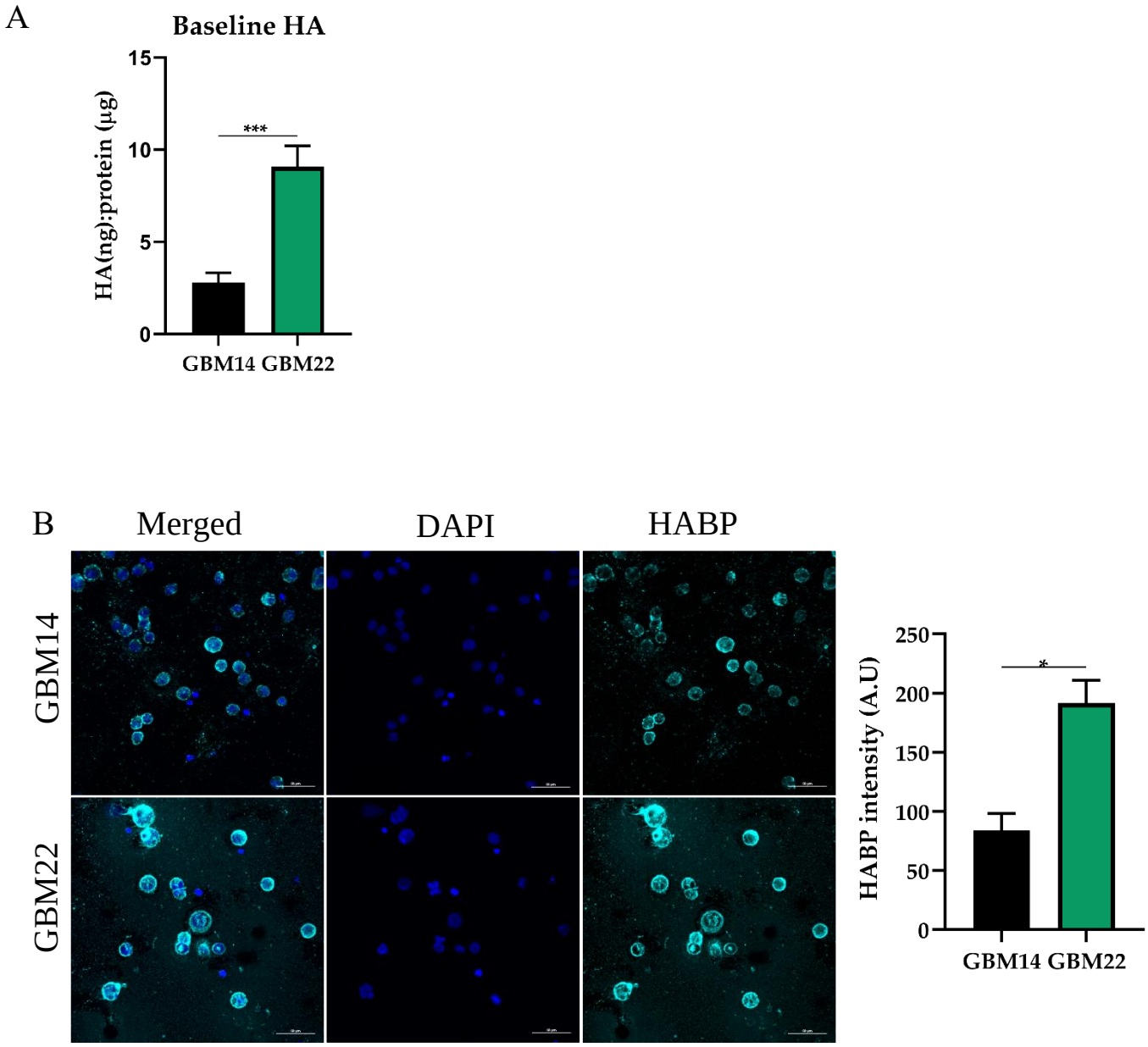


Figure 2.3: Endogenous hyaluronic acid production in GBM cells. (A) Data from a direct ELISA show significantly higher levels of hyaluronic acid (HA) in the media of GBM22 cells compared to GBM14 cells, N=3. (B) Representative images of PDX cells stained with the hyaluronic acid binding protein (HAPB). Quantification of these images shows GBM22 cells have significantly higher HAPB signal on the surface of and between cells than GBM14 cells. Images were acquired with a Nikon A1 air objective 20x, Scale Bar=50µm, N=3.

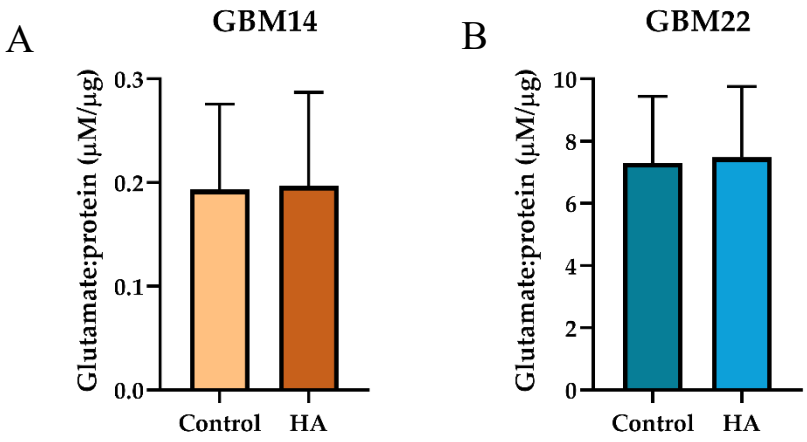


Figure 2.4: Glutamate recovery after HYAL incubation. (A) GBM14 and (B) GBM22 cells were treated with 0.5mg/mL HYAL in Neurobasal medium for 24 hours to remove hyaluronic acid. The media was then replaced with either control Neurobasal medium, or medium supplemented with 0.5mg/mL HA for an additional 24 hours. No difference in extracellular glutamate concentration was observed between control and HA-supplemented conditions.

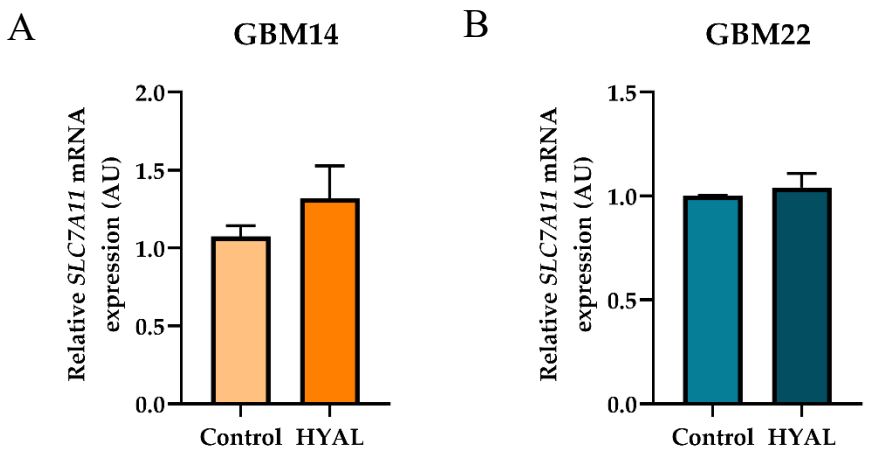
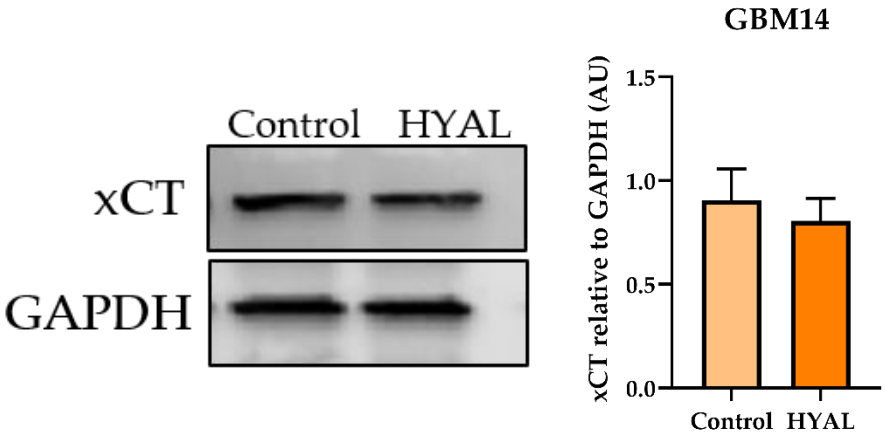


Figure 2.5: Hyaluronidase does not regulate xCT at the transcriptional level. *SLC7A11* transcript expression was unchanged after a 24 hour incubation with 0.5mg/mL HYAL compared to control media in (A) GBM14 and (B) GBM22 cells, N=3.

A



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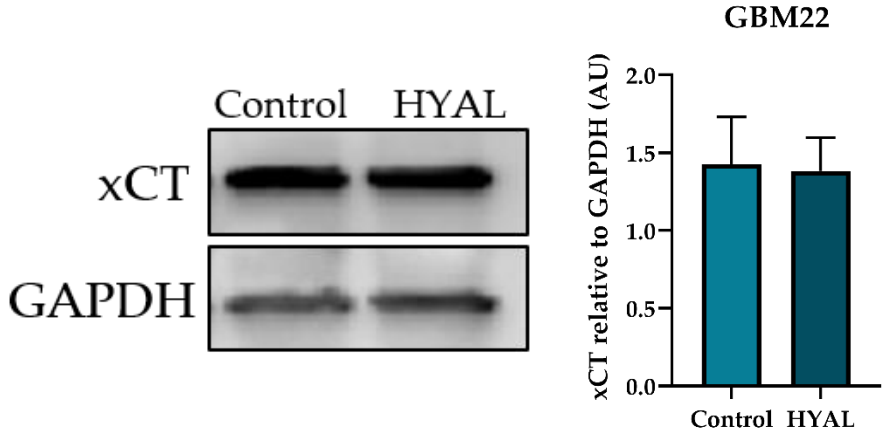

Figure 2.6: Hyaluronidase does not regulate xCT at the translational level. Whole-cell xCT expression in (A) GBM14 and (B) GBM22 cells was unchanged after a 24 hour incubation with 0.5mg/mL HYAL compared to control media as determined by quantification of xCT band intensity relative to GAPDH, N=3.

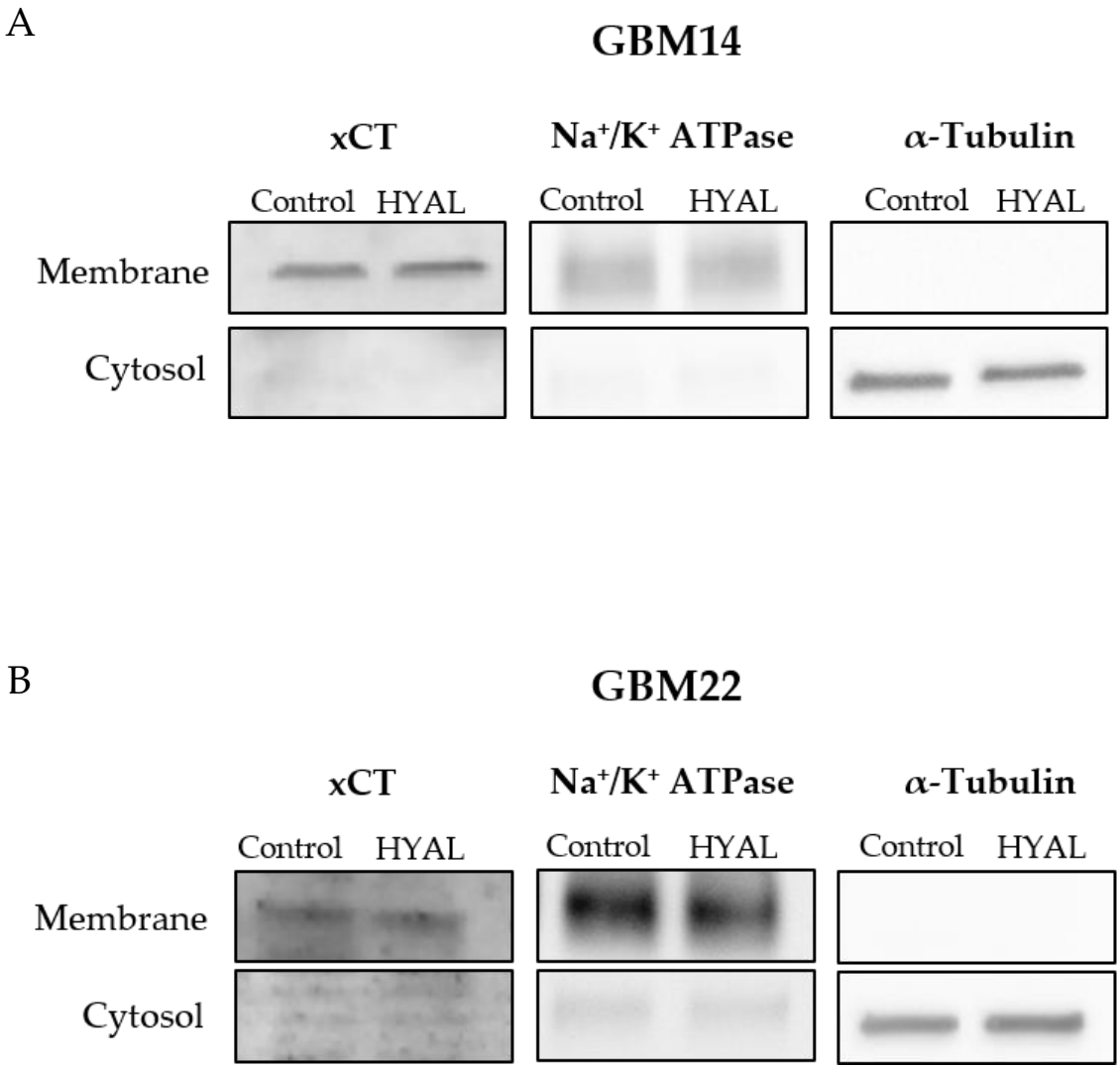

Figure 2.7: Isolation of membrane-bound xCT in PDX cells. We observed no difference in membrane-bound xCT in (A) GBM14 or (B) GBM22 cells after 24 hour exposure to control media or 0.5mg/mL HYAL. We also probed for the sodium-potassium ATPase as a membrane protein marker and alpha-tubulin as a cytosolic marker to ensure that the sample fractions were pure.

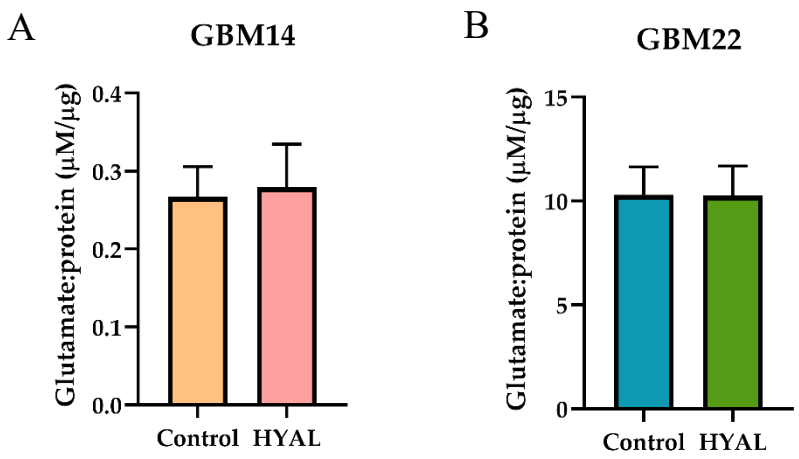

Figure 2.8: Extracellular glutamate concentrations in PDX cells. HYAL treatment (0.5mg/mL, 24 hours) did not alter glutamate release in either (A) GBM14 or (B) GBM22 cells, N=3.

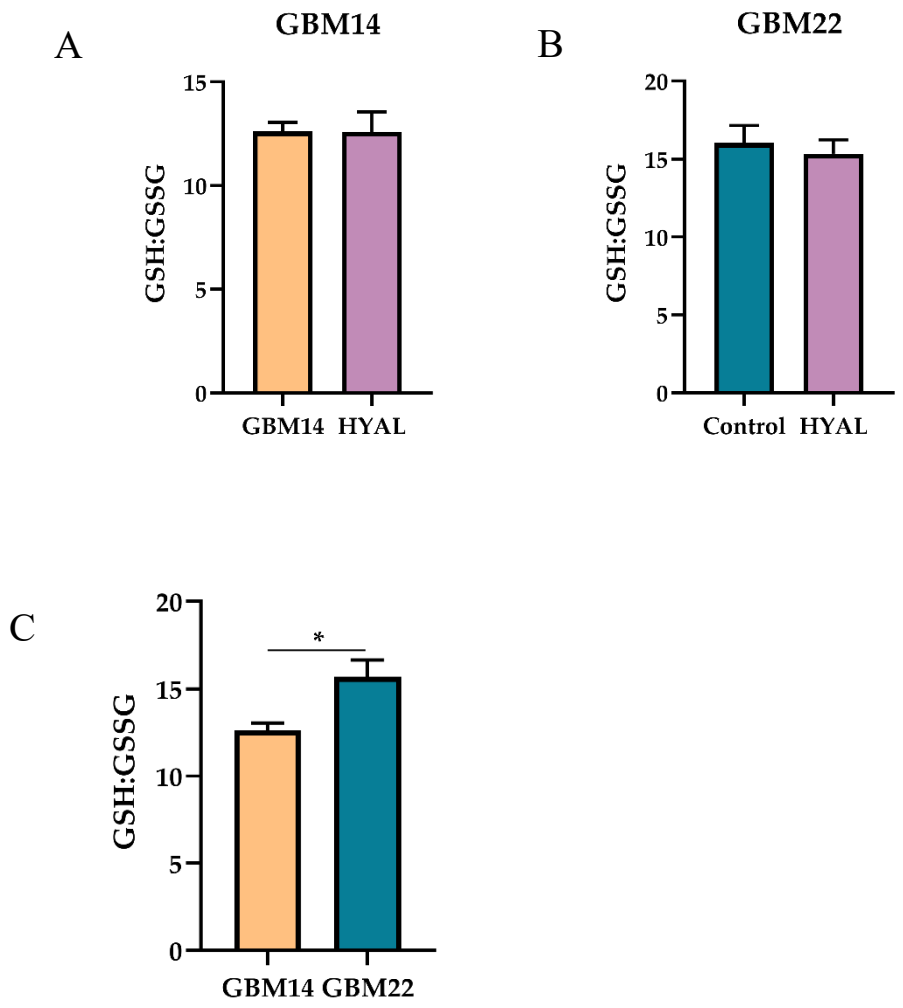

Figure 2.9: Intracellular glutathione concentrations in PDX cells. Intracellular GSH levels were unchanged in response to HYAL treatment (0.5mg/mL, 24 hours) in (A) GBM14 and (B) GBM22 cells, N=3. (C) Intracellular GSH levels were significantly higher in GBM22 cells compared to GBM14 cells due to increased xCT expression and activity in GBM22 cells, N=3.

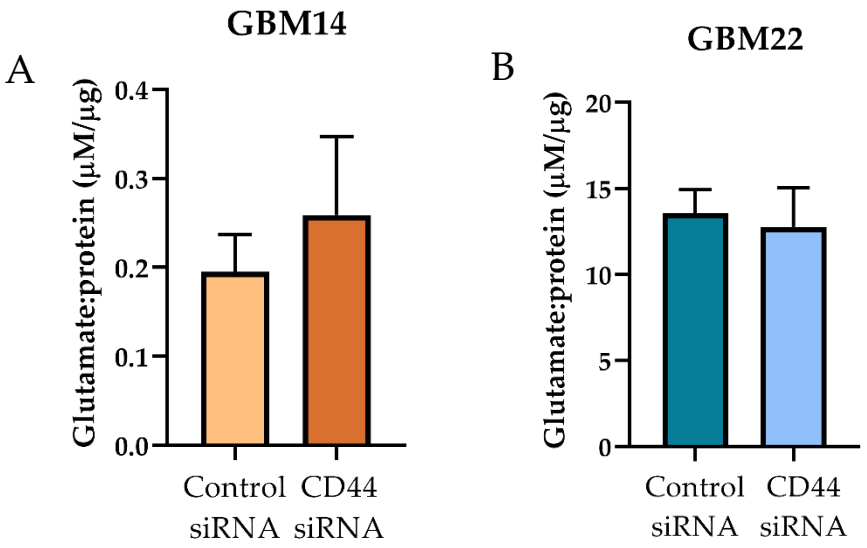

Figure 2.10: The effect of CD44 knockdown on glutamate release and CD44 variant expression in GBM cells. PDX cells were treated with CD44 targeted siRNA for 72 hours, which did not change extracellular glutamate concentrations in (A) GBM14 or (B) GBM22 cells, N=3.

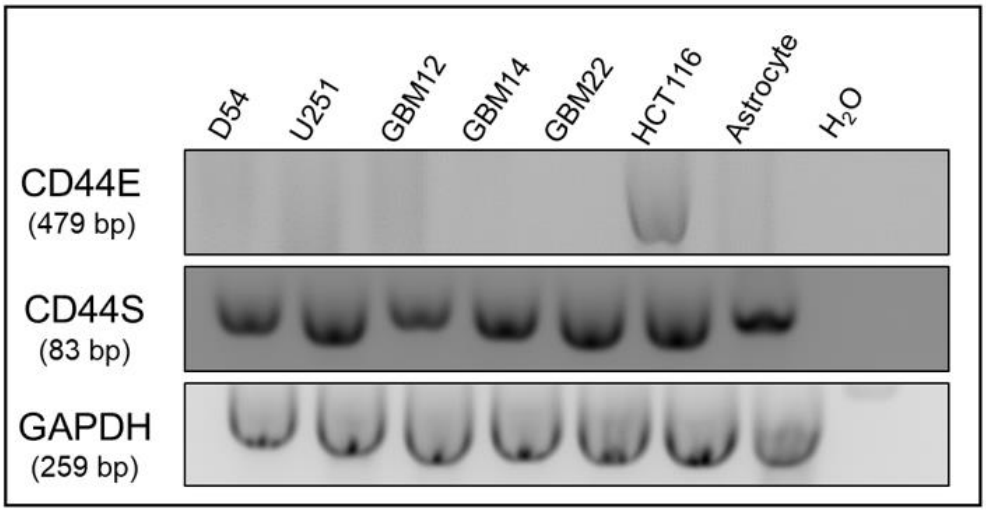

Figure 2.11: Polymerase chain-reaction of CD44S and CD44E expression. RT-PCR of GBM cell lines (D54, U251, GBM12, GBM14, and GBM22), a human gastrointestinal cancer cell line (HCT116) and human astrocytes, showing that brain cells express CD44S transcript, but only HCT116 cells expressed both CD44S and CD44E.

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CHAPTER THREE: EGFR-DEPENDENT REGULATION OF SYSTEM XC- IN GLIOBLASTOMA MULTIFORME

Joelle Martin and Harald Sontheimer

Abstract

GBM is the most common and malignant adult brain tumor. Upon diagnosis, GBM patients have a median surivival time of 12-15 months. One contributing factor to this short survival time is the presence of the membrane-bound amino acid transporter System x_c- (SXC). SXC is responsible for mediating the equimolar exchange of extracellular cystine for intracellular glutamate, and is upregulated in approximately 50% of GBM patients. On average, GBM patients with upregulated SXC expression have shorter survival times and a higher incidence of tumor-associated epilepsy compared to GBM patients with low SXC expression. The epidermal growth factor receptor (EGFR) is the most frequently mutated protein in GBM. Additionally, GBMs harbor a common variant isoform of EGFR (EGFRvIII), which is constitutively active due to a truncated ligand binding domain. Both EGFR and EGFRvIII have demonstrated roles in promoting survival and proliferation of GBM cells, and are the targets of multiple methods of investigational therapies. In this study, we explored EGFR as a candidate regulator of SXC expression and function in patient-derived GBM cell lines, and determined that EGFR plays an important role in regulating aberrant glutamate release mediated by xCT in GBM cells.

Introduction

Glioblastoma is a deadly disease that afflicts thousands of Americans every year [1]. Despite aggressive treatment regimens that include surgical resection, chemotherapy, and radiation, GBM continues to vex physicians and devastate patients. The median survival time after diagnosis is less than 15 months, and the five year survival rate is only 4-5% [2, 3]. These lethal brain tumors have profoundly deleterious effects on a patient's physical, cognitive, and psychological wellbeing [4,

5]. While significant intellectual and financial resources have been spent combating this disease, only marginally effective treatment options such as Bevacizumab and Temozolomide (TMZ) are available for GBM patients. GBM is a highly infiltrative cancer that invades into deep recesses within the brain, further complicating surgical resection and targeted drug delivery efforts [6, 7].

One critical predictor of GBM prognosis is the increased expression of the sodium-independent antiporter system x_c- (SXC) [8]. SXC is comprised of a light chain catalytic subunit (xCT) bound to a heavy chain regulatory subunit (4f2hc/CD98) via a disulfide bond [9]. The xCT subunit is responsible for the equimolar exchange of extracellular cystine for intracellular glutamate at the plasma membrane [10]. Approximately 50% of GBM patients have upregulated levels of xCT expression, and these patients have faster growing tumors and shorter survival times compared to patients with low SXC expression [8, 11, 12]. The excess glutamate exported from xCT creates an excitotoxic environment that kills neurons [8], promotes GBM cell invasion [10], and contributes to the clinical presentation of seizures in up to 60% of patients [4, 13]. Multiple studies demonstrated that inhibiting xCT activity with the drug sulfasalazine (SAS) prolonged survival and decreased seizure incidence in mice, indicating that xCT inhibition is a promising target for GBM therapy development [14-16]. However, phase I/II clinical trials involving SAS as a treatment for GBM ultimately failed due to a lack of efficacy in decreasing tumor burden (likely due to a short biologically active half-life) [17], as well as study participants experiencing adverse events [18, 19]. In theory, pharmacologic inhibition of xCT is promising, but the execution of this therapeutic strategy is more complicated in practice. Therefore, the goal of this study was to identify potential endogenous regulators of xCT expression and function in GBM that may be exploited for treatment purposes.

Epidermal growth factor receptor (EGFR) expression and signaling is indispensable during the processes of development, tissue homeostasis and healing [20]. However, the very pathways necessary for healthy tissues are often corrupted during oncogenesis, and EGFR mutations have been implicated in several cancers, including non-small cell lung cancer, breast cancer, pancreatic cancer, and GBM [21]. Approximately 50% of GBM patients possess tumors with mutated *EGFR*, which leads to EGFR overexpression, gene amplification, enhanced signaling activity, or any combination of the three [22]. Notably, EGFR mutations are more commonly detected in primary GBMs than secondary GBMs [23]. Amplification of the *EGFR* gene is one of the most frequent mutations found in GBM (30-40%) and of those tumors, 50-60% also harbor a variant isoform of EGFR known as EGFRvIII, a novel mutation preceded by EGFR amplification [24]. EGFRvIII lacks a ligand-binding domain due to the in-frame deletion of exons 2-7, and the resulting protein remains constitutively active even in the absence of binding to EGF or other growth factors [25, 26].

Our study utilized two GBM patient-derived xenograft (PDX) lines that represented high (GBM22) and low (GBM14) xCT expression. The goal of our study was investigate the role of EGFR in regulating xCT in GBM PDX cells. To this end, we were able to confirm that EGFR regulates xCT expression and function in GBM cells that highly express EGFR.

Methods and Materials

Cell culture

GBM patient-derived xenograft (PDX) cells were a generous gift from Dr. Yancey Gillespie (Brain Tumor Tissue Core, University of Alabama at Birmingham, Birmingham, AL, USA). Our PDX lines (GBM14 and GBM22) were maintained by serial passage in athymic nude mice, as previously described [27]. Briefly, tumor tissue was harvested 14-21 days post-injection and the

tissue was harvested, homogenized, and approximately 200µl of tissue was injected subcutaneously into the flank of athymic nude mice for tumor propagation. The remaining tissue was suspended in RPMI 1600 media supplemented with enzymes A, R and K (Tumor Dissociation Kit, Miltenyi Biotec, Cat# 130-096-730), and later dissociated mechanically with a GentleMACS tumor Dissociation Kit. Cells were passed through a sterile 40µm filter and maintained as tumor spheres in DMEM/F12 medium (Thermo Fisher, Cat# 21-041-025) supplemented with 10 ng/ml FGF (Thermo Fisher, Cat# PHG0261), 10 ng/ml EGF (Thermo Fisher, Cat# PHG0311), 2% B-27 Supplement w/o Vitamin A (Thermo Fisher, Cat# 12587010), 250 µg/ml amphotericin (Fisher, Cat# BP264550), and 50mg/ml gentamycin (Thermo Fisher, Cat# 15750060), and incubated in 10% CO_2 at 37°C. All cell lines were tested and free of mycoplasma contamination (VWR, Cat# 10497-508). For glutamate assays, cells were incubated in Neurobasal medium (minus phenol red, ThermoFisher/Gibco, Cat# 12348017), which is prepared the same as DMEM/F12 media with the exception of adding 260mM L-glutamine (ThermoFisher, Cat # 25030081).

Cell culture treatment

Afatinib treatment *in vitro*: PDX cells were incubated for 24 hours with 1µM afatinib (Selleckchem, Cat# S1011). Afatinib was solubilized in sterile DMSO to a stock solution of 10mM, passed through a .22µm sterile filter, and then diluted in Neurobasal medium.

Western Blot

Western blotting was performed as previously described [8] with modifications. PDX cells were lysed in RIPA buffer (final concentration 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris Base, pH 8.0), supplemented at 1:100 with protease (Sigma, P8340) and phosphatase (Sigma, P0044) inhibitor cocktails. Protein concentrations were normalized across samples and 15-45 µg of sample lysate was run on a 4-20% gradient precast SDS-polyacrylamide gel (Bio-Rad Cat# 4561094). Gels were then transferred (semi-dry) onto a

polyvinylidene difluoride (PVDF) membrane (ThermoFisher Cat# 88518) using a BioRad Trans-blot Turbo Transfer System at 1.3 amps and 25 volts for 10 minutes. Membranes were blocked in 5% nonfat dry milk in TBS-T (0.1% Tween-20 in Tris-buffered saline, pH 7.4) for one hour at RT. Membranes were incubated with primary antibodies [goat anti-xct (1:450; Abcam; ab60171), rabbit anti-EGFR (1:400; Novus; AF231), chicken anti-glyceraldehyde-3-phosphate dehydrogenase (1:5000; Millipore Sigma; AB2302), in 5% milk overnight at 4°C, then washed 4x10 minutes in TBS-T. Membranes were incubated with HRP-conjugated secondary antibodies: [rabbit anti-goat (1:1500; Santa Cruz; 2922), goat anti-rabbit (1:5000; Invitrogen; 65-6120), goat anti-chicken (1:2000; Santa Cruz-2901)]. Blots using HRP-conjugated secondary antibodies were developed using Supersignal West Femto (Thermo Fisher, Cat# 34095) and imaged on an Azure Biosystems 500 imager. Band intensity values were generated using FIJI software, and experimental protein values were normalized to GAPDH. Images of western blots are representative of 3 independent replicates.

RNA isolation, RT-PCR, and Quantitative RT-PCR

RNA isolation: Cells were plated at 1 million cells in 2ml of media. RNA was isolated from the cell samples using the Purelink RNA Mini Kit (Thermo Fisher, Cat# 12183025). cDNA was then synthesized (stock concentration 50 ng/µL) using SuperScript VILO Master Mix (ThermoFisher, Cat# 11755500) at (25˚C for 10 minutes, 42˚C for 1 hour, 85˚C for 5 minutes, then stored at -20˚C).

qRT-PCR: Expression of *SLC7A11, EGFR,* and *GAPDH* was determined using quantitative real-time PCR. Each cDNA sample (100 ng) was amplified using Taqman reagents on an ABI StepOnePlus real-time PCR System. Taqman probes from ThermoFisher were as follows: *SLC7A11:* Hs00921938_m1, *EGFR:* Hs01076090_m1, and *GAPDH:* Hs02758991_g1. The ΔCt

method was used for baseline mRNA expression quantification. *SLC7A11* and *EGFR* values were made relative to *GAPDH*. Data from three technical replicates were confirmed to be under a standard deviation of 25%. Transcript expression levels were confirmed by three independent biological replicates, and each sample was run with three technical replicates.

Colorimetric Glutamate Assay

PDX cells were dissociated with Accutase Solution (Sigma, Cat# A6964) and seeded at 250,000 cells per 0.5 mL of control or experimental media. Cell media was collected after 24 hours, and glutamate concentrations were measured using a glutamate assay kit (Sigma-Aldrich, Cat# MAK004) according to the manufacturer's instructions. The experiment was set up in technical triplicates and each was run in duplicate on the assay plate. Due to the difference in glutamate concentrations from the two cell lines, 50 µL of sample from GBM14 cells and 5 µL of sample from GBM22 cells was used. The difference in sample volumes was accounted for by using the equation provided in the instruction manual. HYAL treated samples (and controls for these experiments) were processed through 10K centrifugal filter devices (Fisher, Cat# MRCPRT010) for 40 minutes at 14,000xg to remove HYAL, which interferes with glutamate readings. Cells were collected in 60 µL RIPA buffer (see western blot) and total protein concentrations for each well were matched to glutamate concentrations to determine glutamate:protein ratios. Data for these experiments is representative of three biological replicates.

Short Interfering RNA (siRNA)

Two Customized EGFR siRNAs were purchased from Dharmacon. EGFR #1 sense sequence: 5'-GCAAAGUGUGUAACGGAAUAGGUAUTT-3' and EGFR #2 sense sequence: 5'-UUGCAUCAUAGUUAGAUAAGACUGCTT-3' were used to knockdown EGFR expression. 5X siRNA buffer (Dharmacon, Cat# B-002000-UB-100) was diluted to 1X in RNase-free water and

used to reconstitute siRNA to 10 µM. Cells were plated at 250,000 per well and transfected using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher, Cat# 13778030), Opti-MEM I Reduced Serum Medium (Thermo Fisher, Cat# 331985062) and antibiotic-free DMEM for the indicated times. 24 hours before the conclusion of the experiment, cell media was switched to Neurobasal (if cell media was to be analyzed for extracellular glutamate concentration). The total time for EGFR siRNA transfection was 72 hours (western blot) and 96 hours (glutamate assay). ON-TARGETplus Non-targeting Control Pool, 5 nmol (GE Healthcare Dharmacon, Cat# D-001810-10-05) was used as a control and reconstituted in the same manner as experimental siRNAs. The final concentration of siRNA was 100 nm siRNA per well. All siRNA experiments utilized three biological replicates.

Proximity Ligation Assay

Visualization of EGFR and xCT interactions was made possible by using the Duolink™ In Situ Red Starter Kit Goat/Rabbit (Millipore Sigma, Cat# DUO92105-1KT), according to manufacturer's instructions. 400K GBM14 and GBM22 cells were plated on Poly-O treated 15mm coverslips (see Immunocytochemistry, Chapter 2) and allowed to attach overnight. Coverslips were briefly washed 2x with sterile PBS and and then fixed with 4% PFA for 15 minutes at RT. After coverslips were washed again with PBS (2x, quick), cell membranes were stained with 5 µg/ml wheat germ agglutinin diluted in PBS for 15 minutes at RT (ThermoFisher Cat# W32466). Coverslips were washed with PBS (2x, quick) and then incubated with PBSTw (PBS with 0.1% Tween-20) for 20 minutes at RT. Coverslips were washed with PBS (2x, quick), blocked with the provided blocking buffer for 1 hour at 37 ˚C, and then incubated at 4˚C overnight with primary antibodies [goat anti-xct (1:100; Abcam, ab60171), rabbit anti-EGFR (1:75; Novus, AF231)]. The rest of the experiment was conducted according to manufacturer's instructions. Images were

acquired using an A1R Nikon Laser Scanning microscope with a Plan Apo 40X/N.A.1.40 oil objective with a 5x zoom. A Nikon spectral detector was utilized separate 550 and 647 signal to limit channel bleed-through. Representative images were selected from data sets containing three biological replicates and analysis consisted of 6-10 cells per condition for each replicate. Nikon Elements software was used for image analysis and quantification. Using wheat germ agglutinin as a reference for cell perimeter, automated ROIs were generated for each cell analyzed. The Spot Detection tool was then used to identify "bright spots" in the 546 channel within each ROI, and were counted in an automated, unbiased fashion. Cell area was also automatically generated by the Nikon software and used to quantify the PLA signal to cell area ratio.

Statistical Analysis

Results are presented as means ± SEM. Significance was defined as $*p < 0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$. GraphPad Prism 8.0.1 was used for statistical analysis. Normally distributed data were subjected to either a one-way ANOVA with Tukey post-test, or a two-tailed Student's *t* test.

Results

EGFR expression in GBM xenograft cells

To study the regulation of xCT expression and function by EGFR, we selected two examples of GBM cells (GBM14 and GBM22) which have low (GBM14) and high (GBM22) expression of *SLC7A11/*xCT (Figure 2.1). We next probed the transcript and protein levels for EGFR in the two GBM xenolines. GBM22 cells with high *SLC7A11*/xCT expression levels showed significantly lower *EGFR* transcript (Figure 3.1A) and EGFR protein expression (Figure 3.1B) compared to GBM14 cells. Because we did not see the positive correlation between EGFR and xCT expression in our cell lines that has been previously reported [28], we probed for EGFR and xCT expression

in ten frozen GBM tissue samples provided by Carilion Roanoke Memorial Hospital. In agreement with our cell line data, no clear relationship between xCT expression and that of EGFR or EGFRvIII was observed (Figure 3.2).

EGFR interacts with xCT in GBM xenograft cells

EGFR is a demonstrated binding partner of xCT in adherent cells [28]. To further probe if the purported interaction between EGFR and xCT exists in GBM14 and GBM22 cells, we utilized a proximity ligation assay (PLA). PLA signal was observed in GBM14 and GBM22 cells (Figure 3.3A,B). We observed significantly higher counts of PLA signal in GBM14 cells compared to GBM22 cells (Figure 3.3C). Additionally, we calculated the ratio of PLA counts to the area of each cell quantified, and found that value to be significantly higher in GBM14 cells with this method of quantification as well (Figure 3.3D). Total PLA signal was observed at low background levels when the cells were incubated with antibody diluent, EGFR, or xCT antibodies alone (Figure 3.4A), and the same trend was found when PLA signal was made relative to cell area (Figure 3.4B). Cells were stained with wheat germ agglutinin (WGA) to visualize the membrane, where the interaction between EGFR and xCT should be strongest. Our data provide further evidence for the xCT-EGFR interaction in GBM cells, and the co-localization of PLA signal and WGA suggests that the xCT-EGFR interaction is concentrated at the membrane, not intracellular compartments.

EGFR regulates xCT expression and glutamate release in GBM cells overexpressing EGFR

After determining that xCT and EGFR interact at the membrane of GBM14 and GBM22 cells, we explored the functional implications of this interaction. We utilized two separate siRNAs targeting EGFR to evaluate xCT expression after EGFR knockdown. EGFR knockdown decreased xCT protein expression 72 hours after transfection, however this was only seen in GBM14 cells, which overexpress EGFR (Figure 3.5A). In contrast to GBM14 cells, we observed no change in xCT

expression in GBM22 cells, although knockdown of EGFR was robust (Figure 3.5B). We next assessed extracellular glutamate concentrations 96 hours post transfection with EGFR siRNA, and found that glutamate release from GBM14 cells was significantly reduced at this time (Figure 3.5C). However, no difference in extracellular glutamate concentration was observed from GBM22 cells 96 hours post-transfection, in agreement with the western blot results (Figure 3.5D).

The regulation of xCT by EGFR is unrelated to tyrosine kinase activity

Lastly, we sought to determine if inhibiting EGFR tyrosine kinase activity modified glutamate release. To test this, we treated GBM14 and GBM22 cells with afatinib, an FDA approved drug most often prescribed to patients with lung carcinomas that harbor EGFR mutations. Compared to earlier iterations of EGFR inhibitors such as erlotinib and gefitinib, afatinib has a higher affinity for the kinase domain of the EGFR protein and irreversibly blocks EGFR signaling [29]. Both PDX lines were treated with 1μM afatinib for 24 hours, and then extracellular glutamate concentrations were measured. No change in extracellular glutamate concentration was observed in either GBM14 (Figure 3.6A) or GBM22 cells (Figure 3.6B), which is in agreement with previous studies showing that gefitinib treatment does not alter glutamate transport in GBM cells [28].

Discussion

Scientists and physicians continually test novel methods for treating GBM at the preclinical and clinical stages, albeit with individual limitations and varied efficacy [30, 31]. EGFR amplification and mutations are a common indicator of poor prognosis in GBM patients, as these genetic aberrations promote angiogenesis, tumor cell survival, and proliferation [32, 33]. While these canonical roles of EGFR are well documented, recent studies have provided interesting insights into non-canonical roles of EGFR in cancer pathogenesis. One example outside of signal

transduction is the discovery of EGFR and EGFRvIII as co-receptors for xCT. Expression of xCT was postitively correlated with that of EGFR in GBM tissue samples, and further investigation determined that xCT physically interacts with EGFR [28]. While our study showed that EGFR and xCT interact, we were unable to corroborate the correlation between EGFR and xCT expression. Future studies would benefit from increasing the number of cell lines used for these experiments, and investigating cells with $EGFR^{high}$ and xCT^{high}, as well as $EGFR^{low}$ and xCT^{low} phenotypes.

Through promoting xCT-mediated glutamate release in GBM cells, EGFR also promotes downstream Ca^{2+} oscillations through activation of the N-methyl-d-aspartate (NMDA) receptor, thus enhancing the migratory and invasive potential of GBM cells [34]. Additional studies found that supplementation of glutamate to the media of GBM cells significantly increased EGFR transcript expression, and showed a predictable increase in cellular proliferation [35]. Taken together, these findings suggest an intricate feedback mechanism by which EGFR promotes glutamate release via xCT, subsequently stimulating Ca^{2+} oscillations through activation of NDMA receptors and promoting continued EGFR signaling through increased EGFR mRNA synthesis.

Recent studies have demonstrated the dynamic nature of the functional relationship between xCT and EGFR. Our study, along with others [28] showed that EGFR expression promotes xCT-mediated glutamate release in GBM cells. The excess glutamate is beneficial to GBM cells, as it promotes invasion as well as destroying nearby neurons [10]. Interestingly, additional studies found that EGFR inhibits xCT activity by indirectly stimulating the phosphorylation of xCT at serine 26 through mTORC2 [36]. This study highlighted the flexibility of GBM cells to react to nutrient availability, and that xCT phosphorylation is decreased in response to glucose deprivation, allowing cells greater access to intracellular GSH concentrations.

First and second generation pharmaceuticals such as erlotinib, gefitinib, afatinib, and dacomitinib target the kinase domain of EGFR, and while useful in combatting cancers such as non-small cell lung cancer, showed limited efficacy against GBM in the clinical arena [37-40]. This may be due in part to the heterogenous expression of EGFR in GBM tissue illustrated in this study and others [41]. Additionally, EGFR missense mutations in GBM occur frequently in the extracellular region of the protein (such as EGFRvIII), rather than in the intracellular tyrosine kinase domain [42]. Preservation of the intracellular domain of EGFR is of particular interest in GBM, as these residues (a.a. 685-1186) are required for the interaction between EGFR and xCT [28]. ATP-binding site inhibitors such as erlotinib and gefitinib block downstream EGFR signaling, but do not appear to interfere with the EGFR-xCT interaction. EGFR inhibitors designed to alter the conformation of EGFR in a way that precludes ATP binding and the interaction with xCT could have the added effect of reducing xCT-mediated excitotoxicity in GBM.

Conclusions

This study provided evidence supporting the role of EGFR in regulating xCT in patient-derived GBM cell lines. We observed that EGFR interacts with xCT in patient-derived glioblastoma cells expressing high levels of EGFR. After knockdown of EGFR through siRNA, xCT levels and extracellular glutamate concentrations were reduced in GBM14 cells. Future studies regarding the mechanism by which glutamate levels are reduced in response to EGFR inhibition are warranted, as EGFR inhibitors are currently being tested as a combinatorial therapy for GBM. This relationship may be exploited to devise novel therapeutics for patients with GBM cells overexpressing xCT, and may be an unexpected way to modulate tumor-associated epilepsy.

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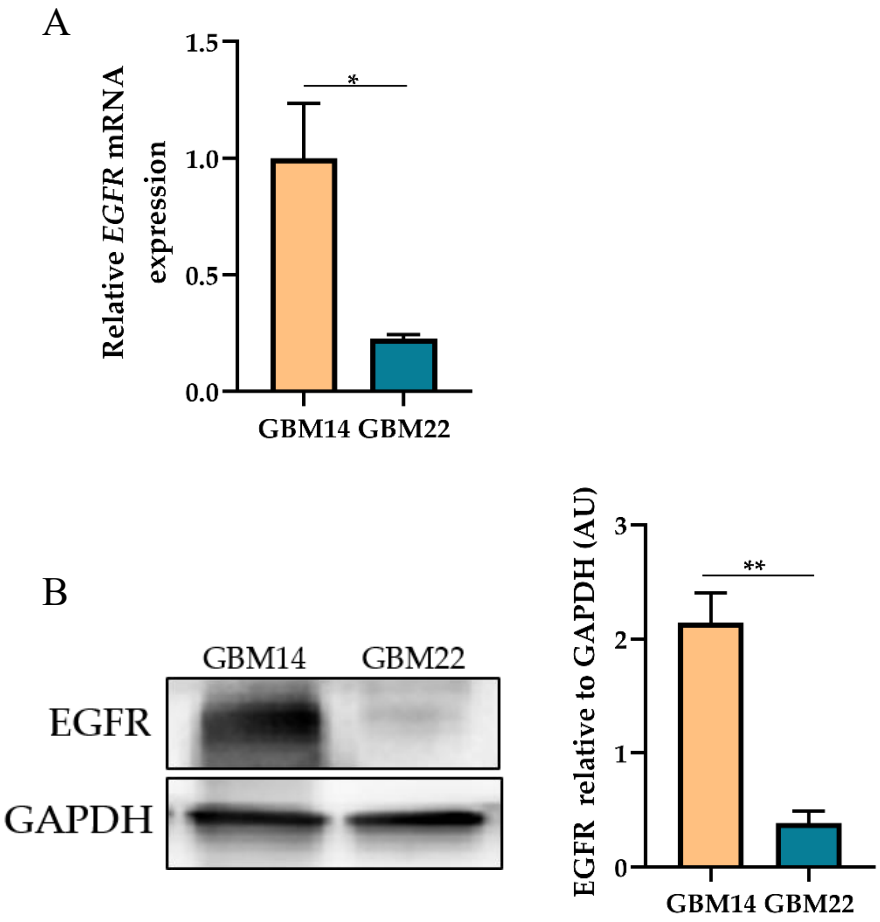


Figure 3.1: Baseline expression of EGFR in two GBM patient-derived xenograft cell lines. (A) *EGFR* transcript expression relative to GAPDH, N=3. (B) Representative western blot of EGFR expression in PDX cells and quantification of EGFR band intensity relative to GAPDH, N=3.

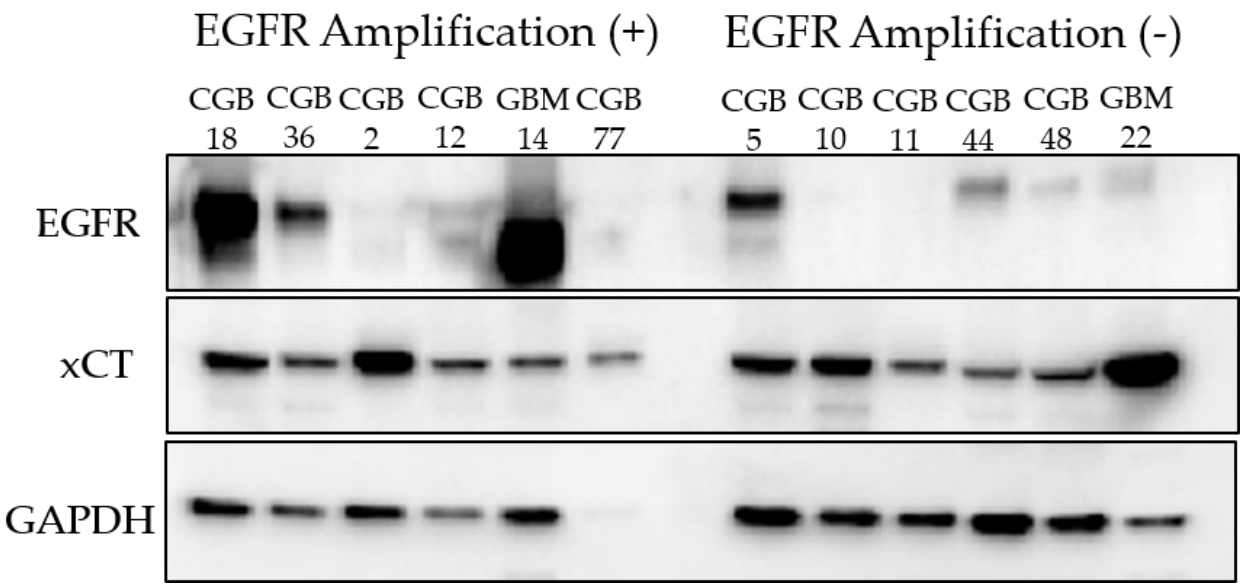
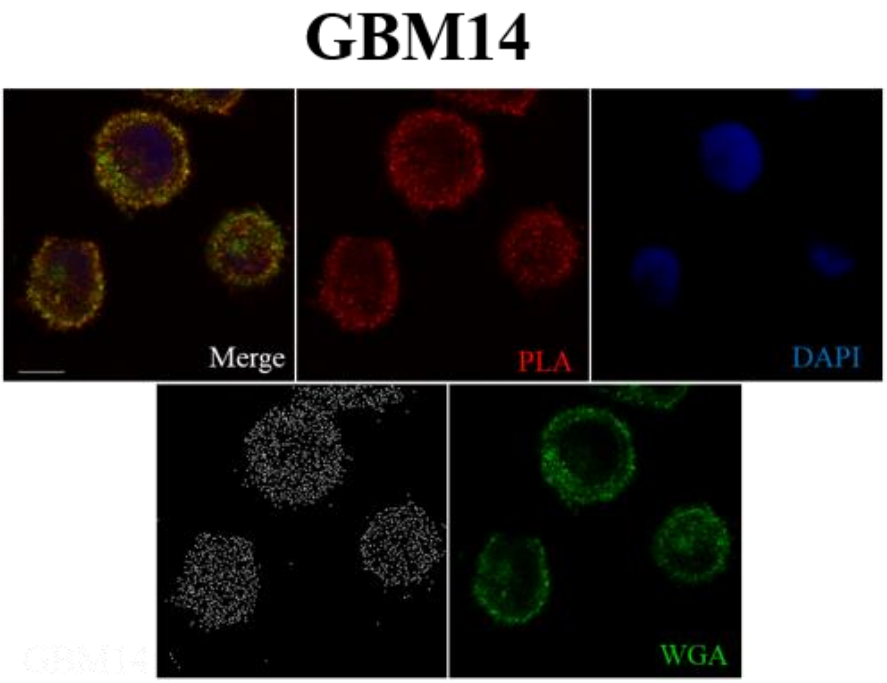
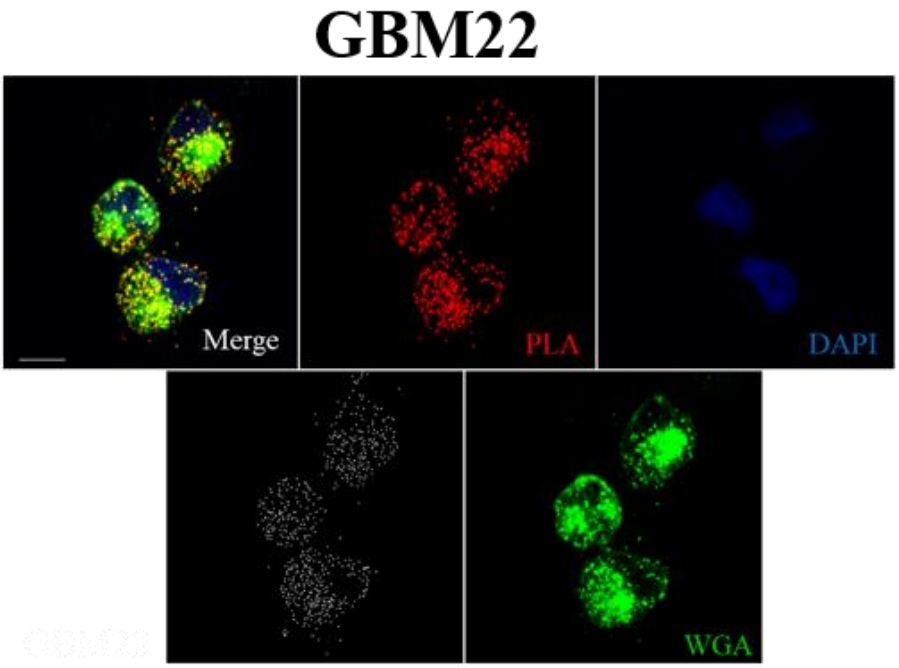


Figure 3.2: EGFR and xCT expression in PDX cell lines and primary GBM tissue. No clear relationship between EGFR and xCT expression was found in ten primary tissue samples and two GBM cell lines. Additionally, EGFR amplification status (determined through Carilion Roanoke Memorial Hospital) was also an uninformative predictor of xCT expression.

A



B



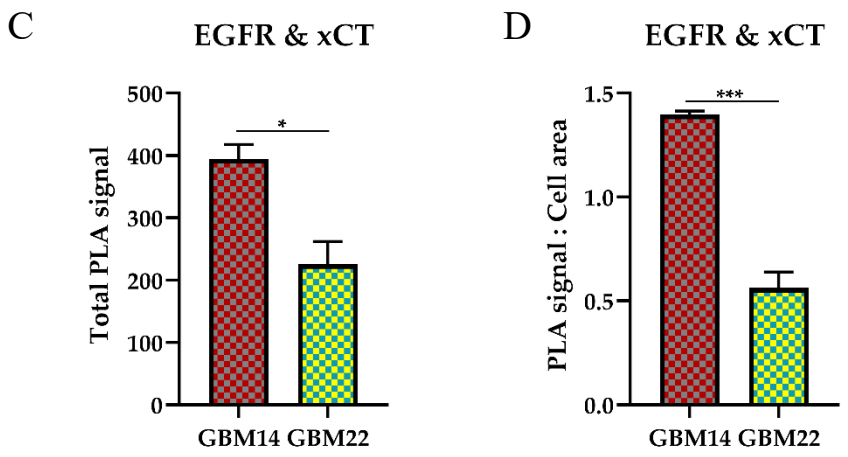


Figure 3.3: EGFR interacts with xCT in PDX cells. (A) GBM14 and (B) GBM22 cells show specific areas of PLA signal (red/white dots), indicating a physical interaction between xCT and EGFR in both cell lines. Images were acquired with a Nikon A1 40x oil objective with a 5x zoom. Scale bar =10µm, N=3. (C) Total PLA signal is greater in GBM14 cells compared to GBM22 cells. (D) PLA signal as a ratio of cell area is greater in GBM14 cells compared to GBM22 cells, N=3. Analysis was conducted using Nikon Elements Software.

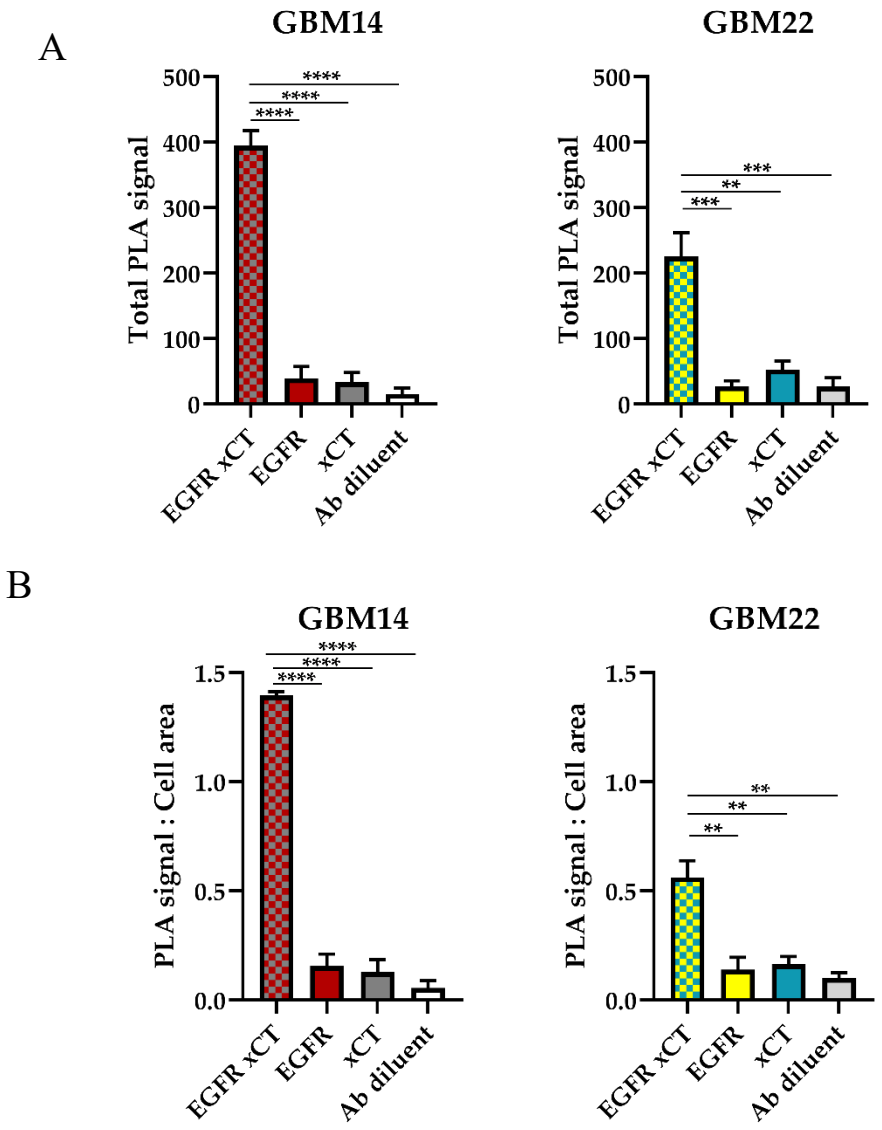


Figure 3.4: Quantification of xCT-EGFR interactions. (A) Experimental PLA signal (EGFR and xCT antibodies) compared to control conditions of antibodies or antibody diluent alone, showing low levels of background detection after counting total PLA signal in GBM14 and GBM22 cells, N=3. (B) Experimental PLA signal (EGFR and xCT antibodies) compared to control conditions of antibodies or antibody diluent alone, showing low levels of background detection after counting the PLA signal as a function of cell size in GBM14 and GBM22 cells, N=3. Analysis was conducted using Nikon Elements Software.

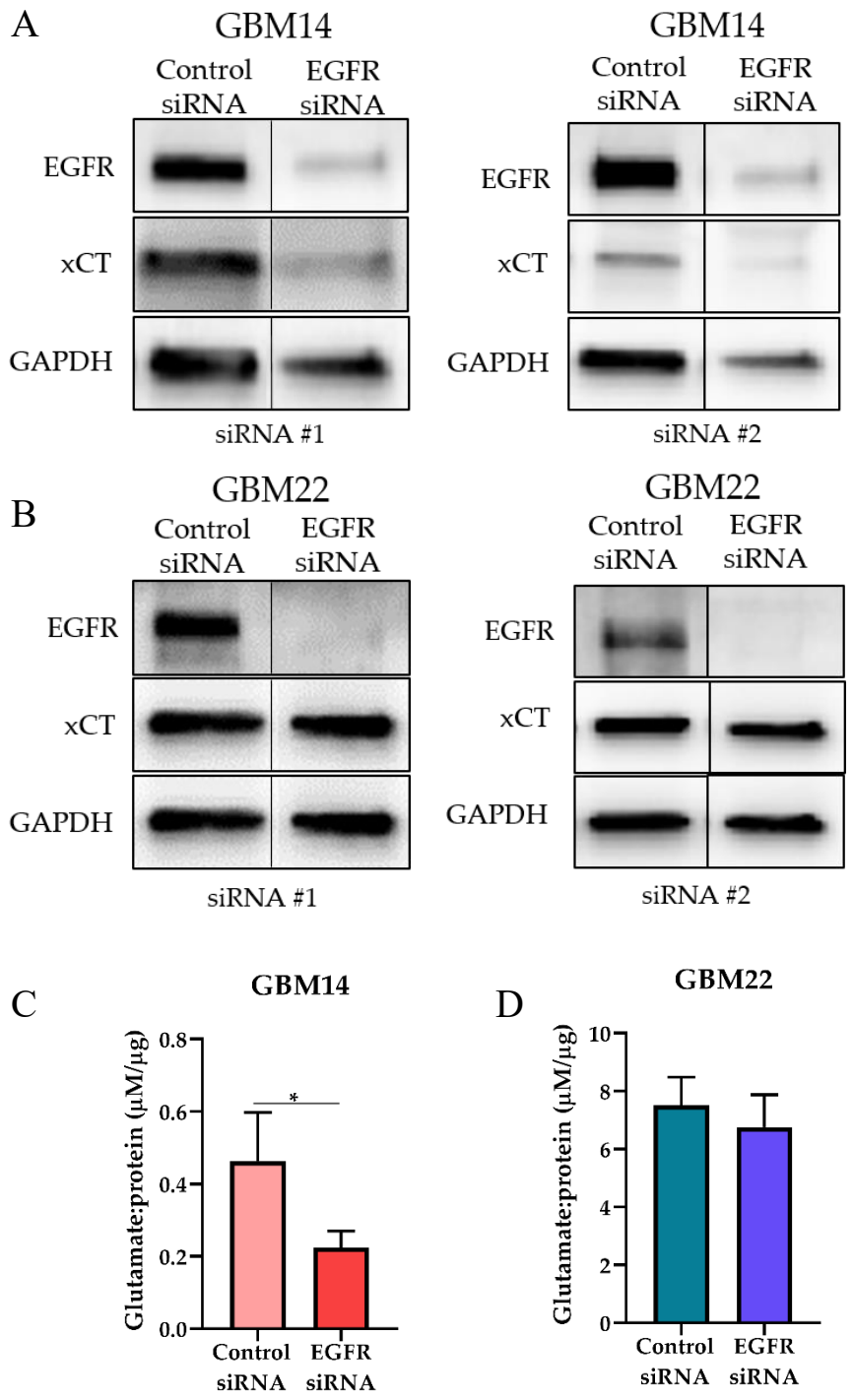


Figure 3.5: The effect of EGFR knockdown on xCT expression and function in GBM14 and GBM22 cells. (A) xCT protein expression is significantly diminished 72 hours post transfection with EGFR siRNA in GBM14 cells, N=3. (B) xCT protein expression is unchanged 72 hours post transfection with EGFR siRNA in GBM22 cells, N=4. (C) A significant decrease in extracellular glutamate release was observed 96 hours post-transfection with EGFR siRNA in GBM14 cells. (D) EGFR siRNA did not change extracellular glutamate concentration in GBM22 media, N=3.

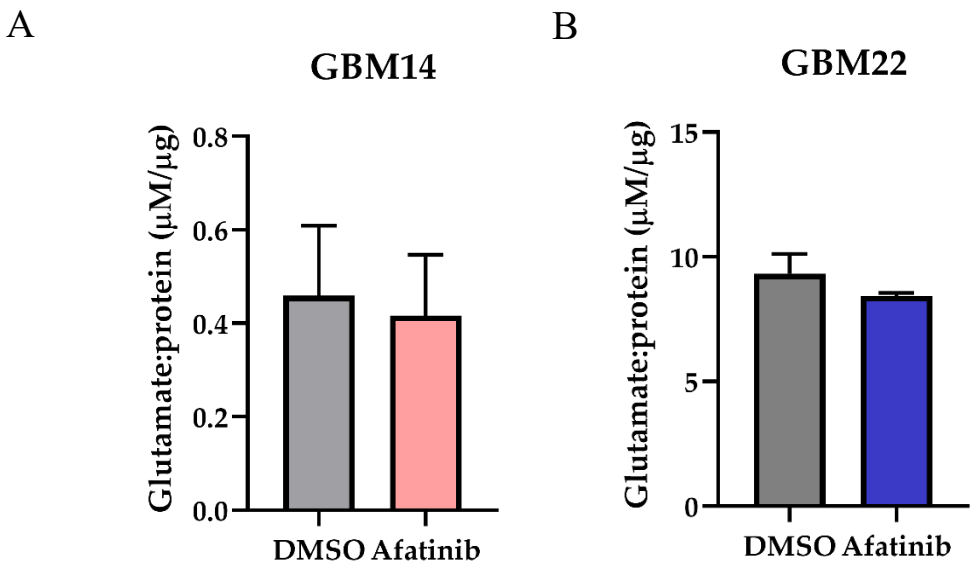


Figure 3.6. The effect of EGFR inhibition on xCT extracellular glutamate release. No difference in extracellular glutamate concentration was observed following 24 hours of the irreversible EGFR inhibitor afatinib (1µM) incubation in (A) GBM14 cells or (B)GBM22 cells.

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CHAPTER FOUR: SUMMARY AND CONCLUSIONS

The rapid growth and invasion of GBM tumors and scarce therapeutic options highlight the critical unmet need for patients afflicted with this disease. As detailed throughout this thesis, the xCT antiporter plays a significant role in GBM disease progression in two ways [1]. First, xCT exports high concentrations of glutamate into the extracellular space, which creates an excitotoxic environment for surrounding neurons and contributes to the presentation of tumor-associated seizures. Second, xCT imports extracellular cystine (cysteine dimer) into the cell, where it is utilized for the production of the antioxidant glutathione [2]. Although pharmacological inhibitors of xCT have shown pre-clinical promise [3-5], no therapies targeting xCT-mediated glutamate transport currently exist for GBM patients.

Broadly, the goal of the studies described in this thesis was to identify molecular regulators of xCT expression and/or function in two patient-derived GBM cells: GBM14 (low xCT expression) and GBM22 (high xCT expression). To this end, two important findings were made. 1) The CD44-HA interaction is not involved in regulating xCT in GBM cells, although CD44 has been implicated in regulating xCT in lung, breast, and gastrointestinal cancers [6], and 2) I was able to confirm the role EGFR in regulating xCT expression and function in GBM cells, specifically cells that express high levels of EGFR. Together, these findings illustrate the diversity of oncogenic pathways between cancers as well as within tumors of the same cancer type.

Clinical perspectives

A common presenting symptom of GBM is the onset of seizures, which is attributed to maladaptive glutamate signaling in the brain, much of which can be attributed to xCT activity [1]. While the canonical functions of CD44 and HA in GBM are to promote cell migration, invasion, and proliferation [7], a recently discovered non-canonical role for CD44 included a stabilizing

interaction with xCT [8]. This interaction, if conserved in GBM, would support glutamate release and therefore seizure induction. The data presented in chapter 2 of this dissertation provided convincing evidence that non-canonical roles of CD44 and HA in GBM do not extend to regulating xCT expression or activity in GBM cells. However, HA appears to have a role in promoting epileptogenesis within the CNS through a different mechanism. Various seizure models have shown that degradation or overall absence of HA leads to seizure induction [9, 10]. Furthermore, degradation of HA surrounding peri-neuronal nets leads to dysfunctional electrical properties of peritumoral neurons. Specifically, peritumoral excitatory neurons become hyperexcitable, and surrounding inhibitory interneurons are either eliminated through excitotoxicity, or their functionality is severely impaired [11]. Taken together, converging lines of research have demonstrated that HA does indeed have an interesting, somewhat unexpected role in fostering the excitotoxic environment fabricated by GBM cells. CD44 was absent on the PNNs of cerebellar neurons [12], however it is possible that HA fragments from digested PNNs could bind to CD44 molecules on more distant neurons, resulting in pro-inflammatory signaling cascades.

EGFR and EGFRvIII are two of the most clinically relevant proteins under current investigation for GBM therapy [13]. EGFR amplification is a predictor of preoperative seizures in GBM patients [14] and is a putative binding partner of xCT [15]. EGFR inhibitors have so far conferred neglible clinical benefit to GBM patients, but EGFRvIII has been identified as a target protein for immunotherapy delivery, as it is a specific marker of cancer cells [16]. The inefficacy of EGFR inhibitors in GBM is partly due to the unique mutations of the protein in the extracellular domain that are less frequent in peripheral cancers [17]. An additional explanation for EGFR inhibition failure in GBM cells is the compensatory signaling pathways including PDGF, insulin-like growth factor 1, and upregulation of other members of the ErbB family of proteins [18, 19].

Based on extensive reviews of clinical trials involving EGFR and other tyrosine kinase inhibitors, it is clear that multimodal approaches are needed for the treatment of GBM, an effort not simplified by the BBB and rapid growth of these tumors.

Concluding Remarks

CD44 and EGFR are two proteins highly implicated in the pathogenesis and continued growth of GBM, both through canonical and non-canonical pathways highlighted in this dissertation. Here, I investigated a previously unexamined role of CD44 and HA in regulating xCT expression and function in GBM. Through several experimental methods, I concluded that these two signaling molecules were not influential in mediating xCT activity in two GBM cell lines. The reason for this appears to be that GBM cells do not express the CD44 variant CD44v8-10 required to interact with xCT. This is a reasonable explanation because CD44v8-10 has low affinity for HA, which has been shown to be instrumental for GBM motility and proliferation [20, 21]. Additionally, I demonstrated that the tyrosine kinase EGFR does interact with xCT in GBM cells and has the capacity to regulate xCT expression and affiliated glutamate transport. This relationship was stronger in GBM14 cells, which have higher expression of EGFR than GBM22 cells. It is possible that GBM cells expressing such high levels of xCT do not require a stabilizing protein, or that a different interacting protein has not yet been identified in this type of cell.

Both findings are important to the field, as determining "dead ends" in regard to potential therapeutics is necessary in order to consolidate ideas and resources toward a more fruitful avenue. Additionally, the relationship between EGFR and xCT is worthy of further investigation. If administered only to patients with tumors expressing high levels of EGFR, EGFR inhibition (and concomitant reduction of xCT-mediated glutamate release) may have a clinical quality of life benefit that has not yet been an analyzed endpoint of previous clinical trials. Future studies

regarding the relationship between CD44 and EGFR would also be informative, as these two proteins are suggested binding partners of one another in head and neck cancer [22]. Based on the current knowledge of the field, discovering a silver bullet for GBM is unlikely. Instead, broad and personalized medicine approaches are necessary to combat this rapidly progressing, heterogeneous disease.

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