UNIVERSITÉ DU QUÉBEC À MONTRÉAL

A TRANSCRIPTIONAL SIGNATURE FOR THE G6PC3 AND SOLUTE CARRIER FAMILY MEMBERS SLC37A2 / SLC37A4 IN THE METABOLIC REPROGRAMMING OF HUMAN GLIOBLASTOMA TUMOURS AND U87 GLIOBLASTOMA-DERIVED NEUROSPHERES

THESIS

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UNE SIGNATURE TRANSCRIPTIONNELLE POUR LES MEMBRES DE LA FAMILLE DES TRANSPORTEURS DE SOLUTÉS G6PC3 ET SLC37A2 / SLC37A4 DANS LA REPROGRAMMATION MÉTABOLIQUE DES TUMEURS HUMAINES DE GLIOBLASTOME ET DES NEUROSPHÈRES DÉRIVÉES DU GLIOBLASTOME U87

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DEDICATION

I dedicate this thesis to my parents Hossein Torabidastgerdooei and Masoome Sadeghi who sacrificed a lot for me to get where I am now.

To my husband Saeed Izadi, who was always present in my ups and downs and never doubted me.

To my beloved sister who supports and encourages me all the time and to my little brother who always has my back.

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ABBREVIATIONS AND ACRONYMS LIST

ABC	ATP-Binding cassette
DNA	Deoxyribonucleic acid
AKT	Cellular homologue of the thymoma virus oncogene akt8
RNA	Ribonucleic acid
ATRX	*Alpha-Thalassemia X-Linked mental retardation syndrome*
BRAF	Homologue of murine sarcoma viral oncogene v-raf B1
cAMP	Cyclic adenosine monophosphate
CD133	Cluster differentiation 133
cDNA	Complementary deoxyribonucleic acid
CPA	Cyclophosphamide
CSC	Cancer stem cells
FN1	Fibronectin 1
G6P	Glucose-6-phosphate
G6Pase	Glucose-6-phosphatase
G6PT	Glucose-6-phosphate transporter
GBM	Glioblastoma
GSD	Glycogen storage disease
GSIS	Glucose-stimulated insulin secretion
IDH	Isocitrate dehydrogenase
mARN	Messenger ribonucleic acid
NANOG	Homeobox protein Nanog
PARP	Poly (ADP-ribose) polymerase 1
RAF	Rapidly accelerated fibrosarcoma
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
SNP	Single Nucleotide Polymorphisms
SOX2	Sex-determining region Y (SRY) - box 2
STAT3	Signal transducer and activator of transcription 3
EMT	Epithelial-mesenchymal transition

TCGA	The Cancer Genome Atlas
TERT	Telomerase reverse transcriptase
TMZ	Temozolomide
TP53	Tumor protein 53
WT	Wild type

UNITS AND SYMBOLS LIST

µM	Micromolar
Gy	Gray
kDa	Kilo Dalton

RÉSUMÉ

Le glycogène, un polysaccharide composé de molécules de glucose, joue un rôle crucial dans le maintien de l'homéostasie du glucose, c'est-à-dire l'équilibre des niveaux de glucose dans l'organisme. En outre, il contribue de manière significative à diverses fonctions clés associées à la survie des cellules cancéreuses du cerveau dans la progression de la maladie du glioblastome multiforme (GBM). L'interaction complexe entre le glycogène et les cellules cancéreuses du cerveau dans les tumeurs hautement hypoxiques nécessite un mécanisme moléculaire adaptatif, qui repose fortement sur la voie glycogénolytique et la détection intracellulaire du glucose-6-phosphate (G6P) par ces cellules. Cependant, l'implication spécifique des composants associés au système de la glucose-6-phosphatase (G6Pase) dans ce contexte reste encore ambiguë et nécessite des recherches plus approfondies afin d'élucider sa contribution précise. Nous avons étudié les niveaux d'expression génétique de divers composants du système G6Pase dans les tissus de GBM, afin de comprendre leur impact fonctionnel sur le contrôle des phénotypes invasifs et des cellules souches cancéreuses du cerveau (CSC). Pour ce faire, nous avons effectué une analyse in silico des niveaux de transcription dans les tissus tumoraux du GBM à l'aide de la plateforme GEPIA. En outre, nous avons extrait l'ARN total et effectué une analyse qPCR pour déterminer l'expression génétique des membres de G6PC1-3 et SLC37A1-4 dans quatre lignées cellulaires différentes de cancer du cerveau humain. Nous avons également utilisé des matrices d'ADNc de tumeurs cérébrales cliniquement annotées pour une analyse plus approfondie. Afin d'évaluer les effets de la transition épithéliale-mésenchymateuse (EMT) induite par le TGF-β et le chimiotactisme cellulaire, nous avons utilisé des techniques d'inhibition transitoire des gènes par siRNA. Pour reproduire le phénotype des CSC du cerveau, nous avons généré des cultures de neurosphères en trois dimensions (3D). Dans le contexte des tissus tumoraux du GBM, il a été observé que le niveau d'expression des gènes G6PC3, SLC37A2 et SLC37A4 était plus élevé que dans les gliomes de bas grade et les tissus sains. En outre, l'expression de ces gènes spécifiques s'est également révélée élevée dans les modèles cellulaires humains de GBM U87, U251, U118 et U138 par rapport aux cellules d'hépatome HepG2. En examinant la relation entre SLC37A4/G6PC3 et SLC37A2, on a découvert que les niveaux de SLC37A4/G6PC3 étaient induits dans les neurosphères U87 3D CD133/SOX2-positives, alors qu'aucun effet similaire n'a été observé dans les monocouches 2D. En outre, il a été observé que l'inhibition de SLC37A4/G6PC3 entraînait des altérations du biomarqueur EMT induit par le TGF-β, le SNAIL, et du chimiotactisme cellulaire. À la lumière de ces résultats, on peut conclure que G6PC3 et SLC37A4, deux composants du système G6Pase, sont associés à la progression de la maladie du GBM et sont impliqués dans la régulation de la reprogrammation métabolique qui se produit dans les phénotypes invasifs et CSC. Il est donc plausible de considérer ces signatures moléculaires comme des cibles potentielles pour de futures interventions thérapeutiques visant à lutter contre la survie des cellules cancéreuses et à renforcer la chimiorésistance.

Mots clés : Glioblastome, Système glucose-6-phosphatase, G6PC3, G6PT, SLC37A2, SLC37A4, Cellules souches cancéreuses, Reprogrammation métabolique

ABSTRACT

Glycogen, a polysaccharide composed of glucose molecules, serves a crucial role in maintaining glucose homeostasis, which is the equilibrium of glucose levels in the body. Additionally, it contributes significantly to various key functions associated with the survival of brain cancer cells in glioblastoma multiforme (GBM) disease progression. The intricate interplay between glycogen and brain cancer cells within the highly hypoxic tumors necessitates an adaptive molecular mechanism, which relies heavily on the glycogenolytic pathway and the intracellular sensing of glucose-6-phosphate (G6P) by these cells. However, the specific involvement of components associated with the glucose-6-phosphatase (G6Pase) system in this context remains shrouded in ambiguity and requires further investigation to unravel its precise contribution. We investigated the levels of gene expression of various components of the G6Pase system in GBM tissues, aiming to understand their functional impact on controlling both the invasive and brain cancer stem cell (CSC) phenotypes. To accomplish this, we performed in silico analysis of transcript levels in GBM tumor tissues using the GEPIA platform. Additionally, we extracted total RNA and conducted qPCR analysis to determine the gene expression of G6PC1-3 and SLC37A1-4 members in four different human brain cancer cell lines. We also utilized clinically annotated brain tumor cDNA arrays for further analysis. In order to assess the effects of TGF-β-induced epithelial-to-mesenchymal transition (EMT) and cell chemotaxis, we employed transient siRNA-mediated gene silencing techniques. To replicate the brain CSC phenotype, we generated three-dimensional (3D) neurosphere cultures. In the context of GBM tumor tissues, it has been observed that there is a higher level of expression in G6PC3, SLC37A2, and SLC37A4 when compared to low-grade glioma and healthy tissue. Furthermore, the expression of these specific genes has also been found to be elevated in established human U87, U251, U118, and U138 GBM cell models in comparison to human HepG2 hepatoma cells. When examining the relationship between SLC37A4/G6PC3 and SLC37A2, it was discovered that the levels of SLC37A4/G6PC3 were induced in 3D CD133/SOX2-positive U87 neurospheres, while there was no similar effect observed in 2D monolayers. Additionally, it was observed that the silencing of SLC37A4/G6PC3 resulted in alterations in the TGF-β-induced EMT biomarker SNAIL and cell chemotaxis. Considering these findings, it can be concluded that G6PC3 and SLC37A4, two components of the G6Pase system, are associated with the progression of GBM disease and are involved in regulating the metabolic reprogramming that occurs in invasive and CSC phenotypes. Therefore, it is plausible to consider these molecular signatures as potential targets for future therapeutic interventions aimed at combating cancer cell survival and enhancing chemoresistance.

Keywords: Glioblastoma, Glucose-6-phosphatase system, G6PC3, G6PT, SLC37A2, SLC37A4, Cancer stem cells, Metabolic reprogramming

CHAPITRE 1

Introduction

1.1 Cancer definition

Cancer, characterized by the anarchic proliferation of cells undergoing neoplastic conversion, stands as a leading health concern, both nationally and globally (Ruddon, 2007). It originates from normally functioning cells after multiple mutational events have taken place, leading to an imbalance in the regulation between cell death and cell proliferation (Ruddon, 2007). This neoplastic transformation often occurs due to carcinogenic chemicals, radiation, or even oncogenic viruses (Čupić, 2012).

In terms of its epidemiological significance, cancer has prominently emerged as the primary cause of death in regions such as Canada and Quebec, with alarming statistics reinforcing this precedence (www.statcan.gc.ca; https://statistique.quebec.ca/fr). In 2022, an estimated 233,900 new cases were diagnosed in Canada, with 85,100 resultant deaths. A closer look into the demographics reveals that this burden is skewed more toward men, accounting for 51.28% of new cases compared to women's 48.7% (Brenner et al., 2022).

1.1.1 Mortality and cancer trends

The gravity of cancer's impact can be further appreciated when examining the mortality rates associated with different cancer types. Data from the WHO's (World Health Organization) GLOBCAN registry in 2018 reflected that lung, liver, pancreatic, esophageal cancer, and leukemia had mortality rates of 84%, 93%, 94%, 89%, and 71% respectively. Alarmingly, barring leukemia, the remaining cancer types boast the lowest survival rates post five years of treatment (Mattiuzzi & Lippi, 2019).

Assessing temporal trends also provides vital insights. White et al., in their analysis of cancer incidence in the USA in 2009, found that cancer cases ascended until the age of 70, post which they saw a decline. This trend was not unique to the US, with similar patterns echoing in Canadian cancer-related death statistics (White et al., 2014). Furthermore, for individuals aged 90 and above, cancer emerged as the predominant cause of death, a statistic expected to surge with an aging population (https://cancer.ca/en/cancer-information/resources/publications/2021-canadian-cancer-statistics) (Figure 1.1).

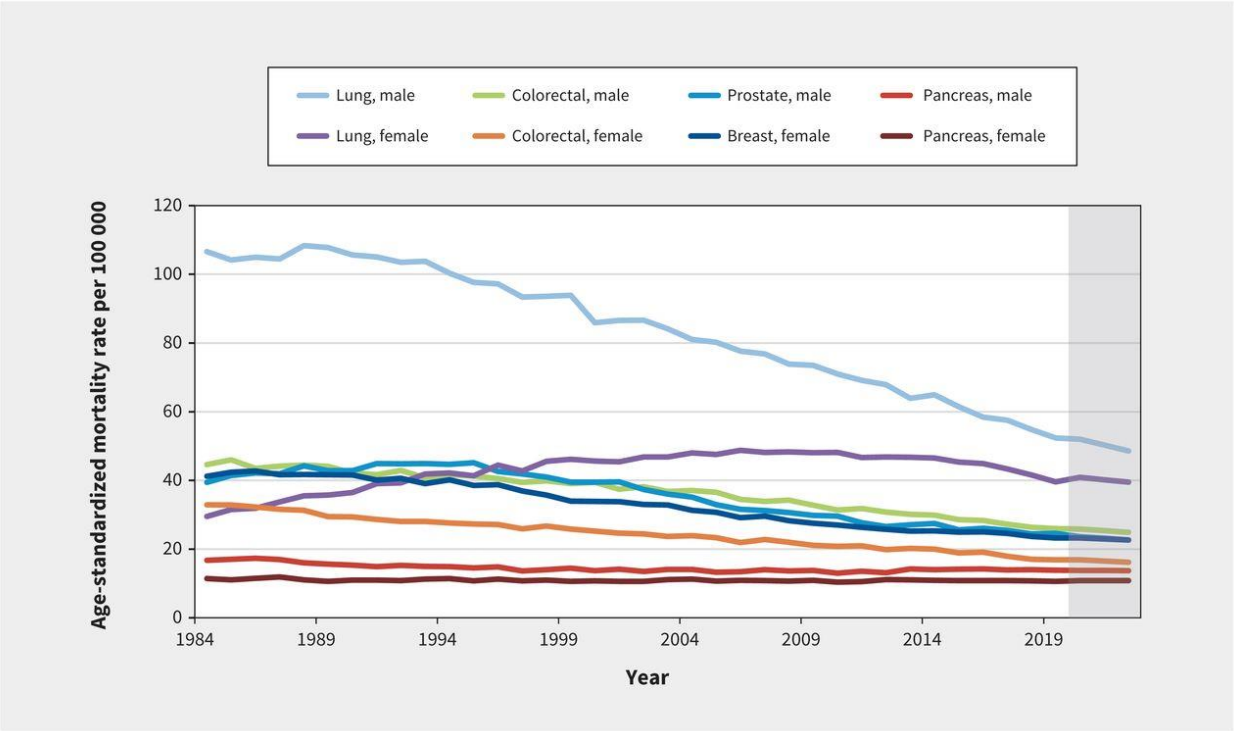

Figure 1.1: Age-standardized mortality rates for selected cancers in Canada, 1984–2022, by sex. Note: Shading indicates projected data (Brenner et al., 2022).

1.1.2 Cancer underlying causes

While the ramifications of cancer are evident, it is equally critical to understand its genesis. The very transformation of a normal cell to a neoplastic one hinges on various factors. Carcinogenesis, or the onset of cancer, stems from widespread changes in gene expression. Such changes disrupt the equilibrium between cell death and proliferation, driving cells towards uncontrollable growth (Ruddon, 2007). Underlying this are carcinogenic agents, such as specific chemicals, oncogenic viruses, or radiation, further triggering and exacerbating the process (Schwab, 2011).

1.1.3 Cancer cell phenotypic variability

A noteworthy aspect of neoplasms is their phenotypic divergence from their non-cancerous counterparts (Rulyak et al., 2003). The landscape of the tumor is not homogenous, thanks to the genetic instability that seeds cellular heterogeneity (Figure 1.2). This diversity endows the neoplasm with aggressive traits, bolstering tumor progression, metastasis, and resistance to therapeutic interventions (Burrell et al., 2013).

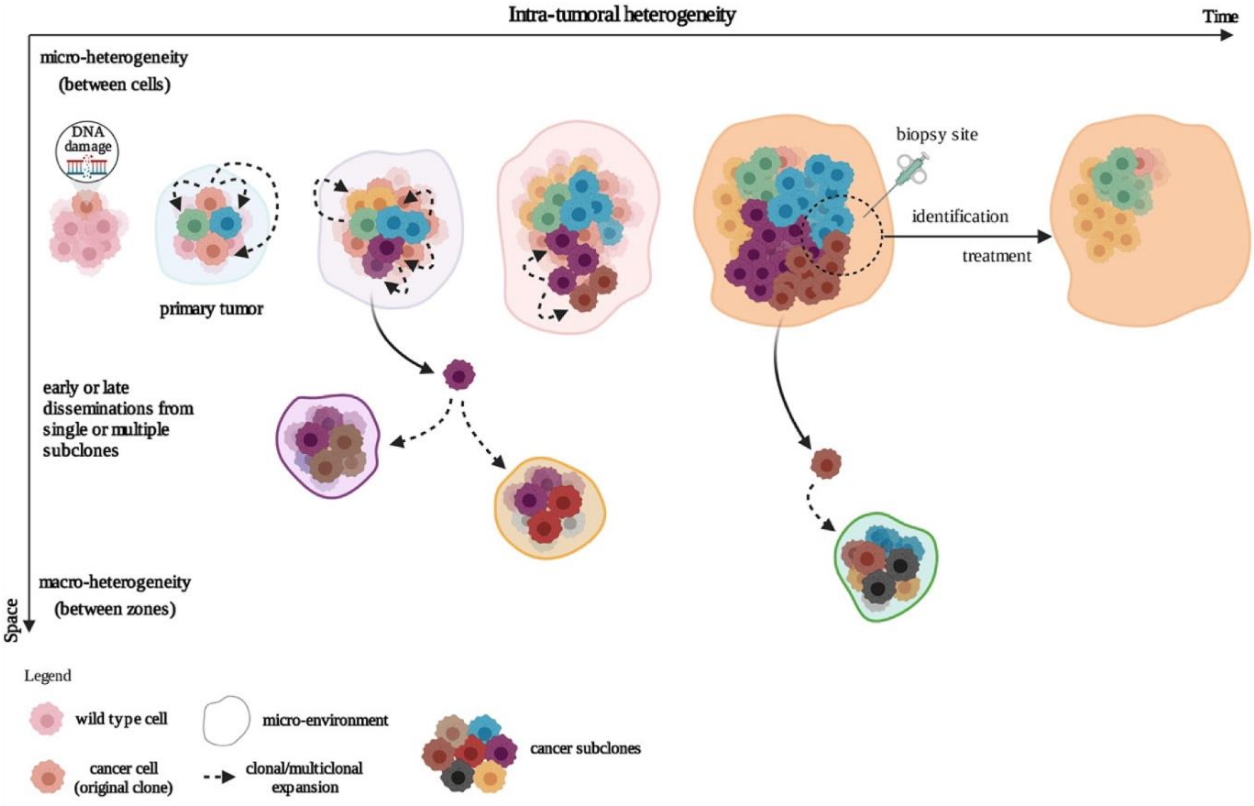

Figure 1.2: Development of intra-tumoral heterogeneity. In the clonal evolution model, tumor cells arise from a single mutated cell and acquire additional varied mutations as they progress. This can occur in a linear fashion (not represented) whereby the cells successively acquire mutations that confer a growth or survival advantage, or through a branched mechanism, giving rise to multiple genetically diverse subclonal populations (Jacquemin et al., 2022).

1.1.4 Metastasis

The gravest stage in cancer's life cycle is arguably metastasis: the transition of tumor cells from their birthplace to distant regions. Utilizing the vascular and lymphatic highways, these rogue cells break free from the primary tumor, embedding themselves into alien tissues or organs (Bacac & Stamenkovic, 2008; Duffy et al., 2008). This relocation culminates in the formation of secondary tumors, amplifying the disease's complexity (Denardo et al., 2008).

1.2 Brain cancer

Brain tumors have gained increasing attention due to a surge in incidence over the past two decades. Men have witnessed a case range from 0.01 to 12.7 per 100,000, while women's incidence lies between 0.01 to 10.7 per 100,000 individuals. Strikingly, northern Europe records the peak occurrence, contrasting the

minimal cases noted in Africa (Mail et al., 2016). An alarming statistic reveals the mortality rate of cancers rooted in the nervous system standing at 3.4 per 100,000 individuals (Khodamoradi et al., 2017).

1.2.1 Origin-based classification

Brain tumors can essentially be bifurcated into two categories: primary and metastatic. While primary tumors find their origin within the Central Nervous System (CNS), metastatic tumors emerge outside of the CNS and can trace their lineage to diverse cancer types like lung, breast, and kidney among others (Fox et al., 2011; Lapointe et al., 2018; Posner, 1996). An intriguing observation states that a significant portion of CNS tumors are identified as gliomas, contributing to 81% of malignant tumors (Lapointe et al., 2018; Ostrem et al., 2014). Gliomas spring from an unchecked proliferation of CNS glial cells, encompassing astrocytes, oligodendrocytes, and ependymata, resulting in specific tumors like astrocytomas and oligodendrogliomas (Jessen, 2004; Ostrom et al., 2016).

The classification landscape of gliomas is not strictly demarcated, inviting various tumor types like medulloblastomas and anaplastic gangliogliomas under its umbrella (Ostrom et al., 2016; Waltz et al., 1991). (Figure 1.3)

1.2.2 Genetic mutation-driven classification

Intriguingly, brain tumors can also be cataloged based on their genetic mutational landscape (Lapointe et al., 2018). Several genes, such as IDH, BRAF, TP53, ATRX, and TERT, have been identified as culprits in tumor genesis due to their mutated forms affecting critical cellular functions (Koh et al., 2004; Maeaka & Janku, 2018; Lapointe et al., 2018; Park et al., 2017; Podlevsky & Chen, 2012). For instance, mutations in the IDH gene meddle with cellular metabolism kickstarting carcinogenesis (Han et al., 2020), while the BRAF gene's mutated form can derail cell differentiation pathways (Matallanas et al., 2011).

The WHO has offered a practical stratification of gliomas based on the growth speed of the tumorous cells, segmenting them into grades I to IV (Weller et al., 2015; Louis et al., 2021). A distinctive trait of Grade I gliomas is their circumscribed nature, offering clearer boundaries, making surgical interventions feasible (Qin et al., 2014). Grades II to IV are, however, termed diffuse, due to their lack of clear demarcation, rendering surgical removal challenging. Genetic mutations, such as those in the IDH, TP53, and ATRX genes, are frequent in these grades, affecting the prognosis which can vary from 2 to 12 years (Eckel-Passow et al., 2015; Batista et al., 2016).

Of specific mention are grade IV gliomas, notably glioblastomas (GBMs), which exhibit a typically aggressive nature. GBMs can either possess the IDH mutation, signifying a transformation from a lower-grade tumor, or lack it, denoting a primary GBM (Ohgaki et al., 2004). The presence or absence of specific mutations, such as in the TERT gene or the IDH gene combined with 1p/19q codeletion, can be instrumental in determining the prognosis of these tumors (Eckel-Passow et al., 2015).

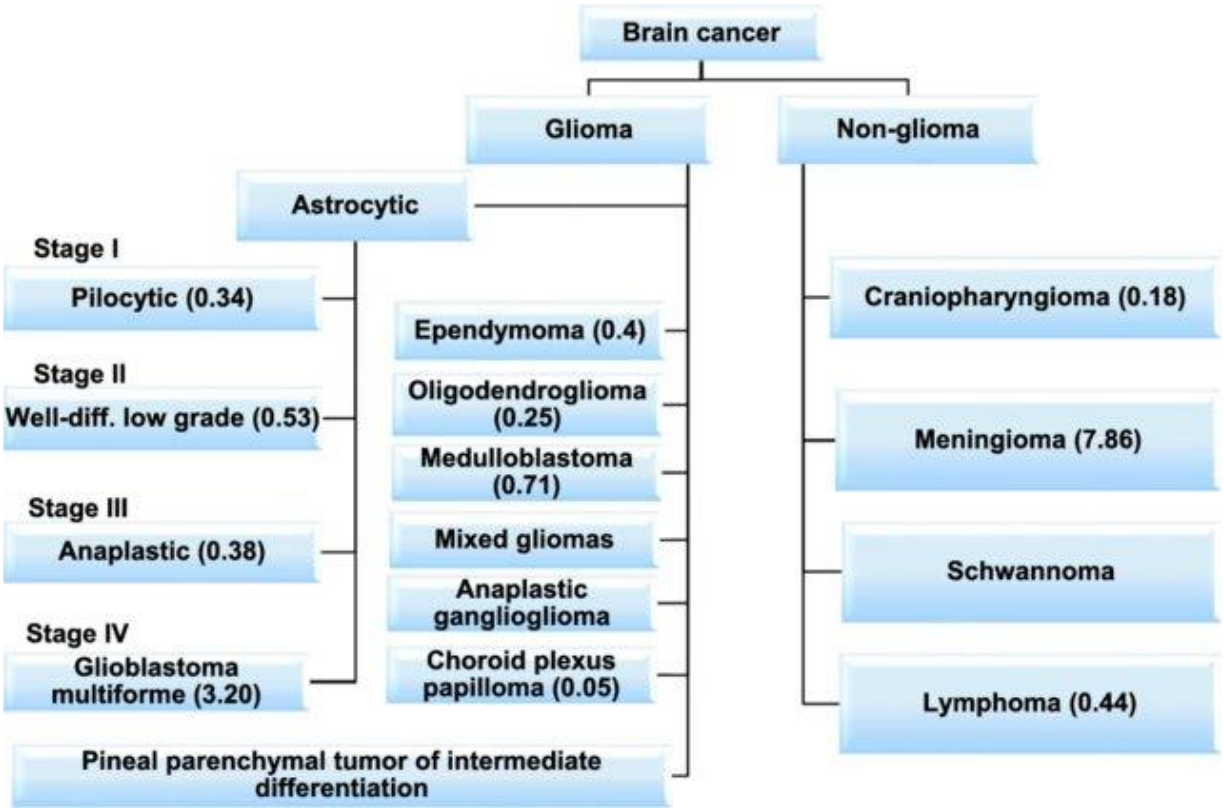

Figure 1.3: Classification of brain tumors as reported from the Central Brain Tumor Registry of the United States (Ostrom et al., 2016). Numbers in parentheses indicates incidence or cases per 100,000 individuals and are age-adjusted to the 2000 United States standard population (Sher- galis, A. et al., 2018).

1.3 Glioblastoma

Glioblastomas (GBMs) stand as the pinnacle of aggressiveness among gliomas, classified as grade IV tumors (Tamimi & Juweid, 2017). With only a 5% 5-year survival rate, their occurrence is frequently seen in distinct lobes such as frontal, temporal, parietal, and occipital regions (Chakrabarti et al., 2005). Predominantly affecting those between 65 and 84, its incidence drops significantly after 85, with the primary GBMs median diagnostic age hovering around 64 and secondary glioblastomas at 40 (Ostrom et

al., 2013). Moreover, GBM seems to have a predilection for the male population in the USA (Thakkar et al., 2014).

1.3.1 Cellular characteristics and origins

These tumors are a convoluted assemblage of three primary cell types: neural stem cells, their astrocytic derivatives, and oligodendrocyte precursor cells (Figure 1.4). Interestingly, GBMs share remarkable resemblance in genetic profiles and cell surface markers with their normal CNS counterparts (Yao et al., 2018). Numerous studies have reinforced the notion that CNS cells, including neural stem cells and their derivatives, can potentially transform into GBMs (Zhu et al., 2005; Chow et al., 2011; Lui et al., 2011; Yao et al., 2018). In essence, it is suggested that the very origin of GBMs lies in the neuroepithelial cells or neural stem cells (de Chevigny and Lledo, 2006). Recent research has highlighted the significant correlation between GBM cells and normal subventricular zone tissue, underscoring the potential role of neural stem cells in GBM onset (Lee et al., 2018). This is further supported by studies that have identified neural stem cells in the subventricular zone as the potential cells of origin for GBM (Zhang, 2021; Loras, 2023; Kwan, 2018; Matarredona, 2019). These findings have important implications for the development of new therapeutic strategies targeting neural stem cells in the subventricular zone.

1.3.2 Pathological traits of GBMs

GBMs exhibit a plethora of pathological features that support tumor advancement. Chief among these are unbridled cell proliferation and mutations promoting cell survival (Greaves & Maley, 2012; Merlo et al., 2006). Cellular anomalies, such as hyperchromatic chromatins and altered nucleus/cytoplasm ratios (surface ratio), are common (Schult et al., 2005). Further, they manifest an increased vascularization marked by morphologically unique vessels, which, in turn, contribute to a disrupted brain-spinal cord barrier and potential for extravasation (G Linkous & M Yazlovitskaya, 2011; Rojiani & Dorovini-Zis, 1996; Ballabh et al., 2004). Necrotic regions also prominently feature in GBMs, either centralized or scattered across the tumor, with the latter surrounded by cellular dense areas termed pseudo-palissades (Kleihues, P. et al., 2000; Brat, D. J. et al., 2004).

1.3.3 Factors impacting GBM occurrence

Multiple factors intertwine in the incidence of GBMs. While environmental components, including lifestyle or consumption habits, currently have no substantiated links to GBMs, demographics play a considerable role. Age, gender, and ethnicity disparities exist in GBM occurrence, with Caucasian individuals showing a

two-fold increased risk compared to other ethnic groups (Thakkar et al., 2014). The two primary variants of GBMs - primary and secondary - exhibit different age-associated onsets and molecular characteristics (Vleeschouwer, 2017; Maher et al., 2006).

In sum, understanding GBMs necessitates a deep dive into their cellular origins, pathological manifestations, and influencing factors. Their aggressive nature underscores the urgency for more efficacious treatments. While several advancements have been made in determining the underpinnings of these tumors, more in-depth research is paramount.

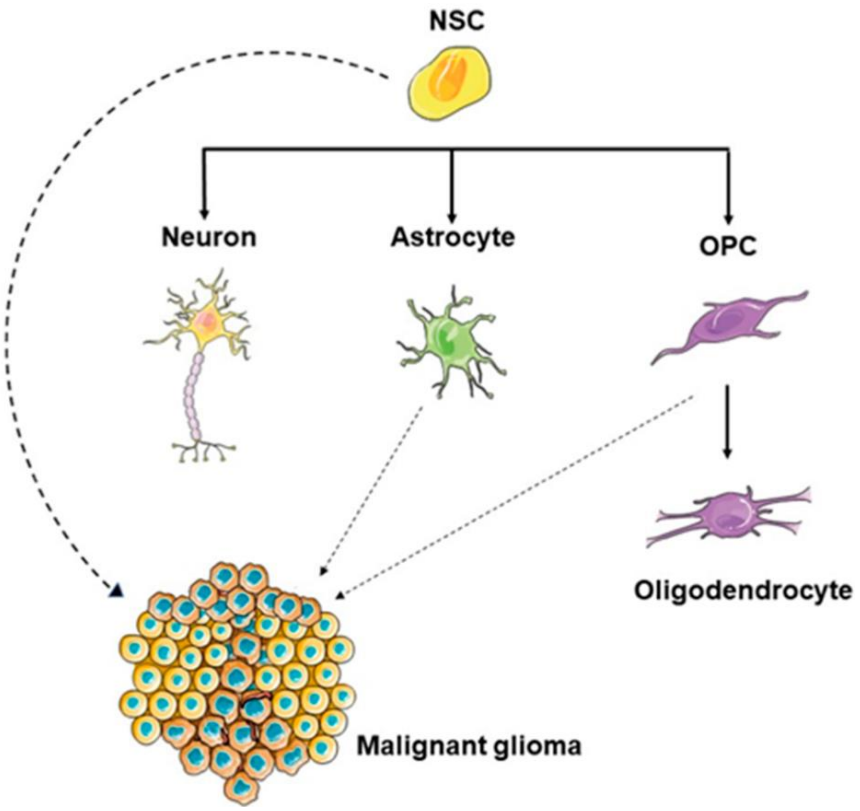

Figure 1.4: The glioma cell of origin. NSCs are undifferentiated cells with self-renewal and multipotent capacities. They give rise to neurons, astrocytes, and OPCs. NSCs can be mutated and converted into glioma stem cells (GSCs) (Loras et al., 2023).

1.3.4 Glioblastoma diagnostic approaches

1.3.4.1 Clinical indications for GBM diagnosis

Patients with GBM often present with a myriad of symptoms. Common warning signs include behavioral changes, visual disturbances, and persistent headaches. More specific symptoms like nausea, vomiting, and impaired alertness hint at intracranial hyperpressure. These often precede neurological symptoms such as seizures (Health Canada).

Neuroimaging plays a crucial role in accurately diagnosing glioblastoma, with radiological examination offering various possibilities including high-grade glioma, abscess, or cerebral lymphoma, while magnetic resonance imaging is valuable in differential diagnosis and histological analysis remaining the gold standard for confirming GBM presence (Weller, 2011, and Ducray and Guillevin, 2007).

1.3.4.2 Radiological and histological insights

The primary tool for GBM diagnosis is neuroimaging, which identifies intracranial lesions. CT scans frequently show a heterogeneous mass suggestive of a high-grade glioma, abscess, or cerebral lymphoma. Magnetic resonance imaging (MRI) aids in differential diagnosis, but histological examination is crucial for accurate diagnosis (Weller, 2011). According to the WHO's 2000 classification, gliomas are categorized based on morphological and histological criteria. GBM, characterized as grade IV, presents poorly differentiated cells, high cell density, and increased mitotic activity (WHO, 2000).

1.3.4.3 Molecular diagnostic techniques

One challenge with diagnosing GBM lies in the potential variability and subjectivity of interpreting certain classification criteria. Hence, molecular diagnostics have emerged to provide unequivocal evidence. Notably, primary and secondary GBMs present different sets of genetic alterations. These could range from chromosomal macro-deletions to specific gene mutations. For instance, mutations in the EGFR (epidermal growth factor receptor) and PTEN (phosphatase and tensin homolog) genes were initially considered as prime molecular markers for primary GBMs. With the evolution of diagnostic techniques, molecular diagnostics now integrates a combination of immunohistochemistry and sequencing tests to provide more specific glioma cell type and grade classification (adapted from WHO classification of gliomas, 2016; Brouland and Hottinger, 2017; and cIMPACT-NOW consortium recommendations).

1.3.5 Glioblastoma therapeutic approaches

The primary aim of managing glioblastoma (GBM) is to achieve a comprehensive eradication of tumor cells, which can be accomplished through various therapeutic modalities including surgical resection, chemotherapy, and radiotherapy. Chemotherapy involves the use of drugs that disrupt the DNA of tumor cells, thereby impeding their growth and proliferation, while radiotherapy directly targets and eliminates cancerous cells. Surgical resection, on the other hand, entails the removal of the tumor mass along with some surrounding healthy tissue to prevent further growth and spread of the cancer.

Research has shown that GBM cells, particularly those exposed to conventional therapies, can enhance immunosuppression, leading to faster tumor growth (Authier, 2015). This immunosuppression is mediated by various factors, including FGL2, which not only suppresses the immune response but also promotes tumor progression (Ma, 2022). The GBM microenvironment also plays a role in immunosuppression, with tumor-derived factors and immunoregulatory leukocytes contributing to the suppression of cellular immunity (Waziri, 2010). Furthermore, the presence of multiple immunosuppressive pathways and an altered natural killer (NK) cell phenotype in GBM further highlight the complexity of the immunosuppressive mechanisms in this type of cancer (Close, 2019).

1.3.5.1 Surgical interventions

Surgical resection is a valuable tool in the management of GBM, offering benefits in both diagnosis and treatment. It can be used to confirm tumor nature, provide insight into genetic mutations, and potentially improve patient prognosis (Lacroix et al., 2001). However, GBMs tend to infiltrate inoperable regions, and despite surgery, median survival is limited, typically ranging from 3 to 6 months (Davis et al., 1998). Thus, surgery is often combined with chemotherapy and radiotherapy to enhance therapeutic outcomes.

1.3.5.2 Chemotherapeutic agents

- **Temozolomide** (TMZ), which gained FDA approval in 2005, acts by alkylating DNA, particularly guanine residues. This leads to cell death when combined with radiotherapy, displaying an increased mean survival duration from 12.1 to 14.6 months (Stupp et al., 2005). ESMO suggests TMZ administration in alignment with 5-day radiotherapy course every four weeks across six radiation cycles (Stupp et al., 2014).

- FDA-approved in 2002, **Carmustine** impedes DNA replication by forming interlinkages between nucleotides. This method elevates median survival periods but might result in cerebrospinal fluid leakage due to brain edema-associated pressure (Westphal et al., 2006).
- **Lomustine**, a nitrosourea alkylating agent, achieves its action by linking nucleotides on the same DNA strand. With its high lipid solubility, it effectively breaches the blood-brain barrier. Recent studies imply that a combination of lomustine and TMZ can boost overall survival times considerably (Herrlinger et al., 2019).
- **Cyclophosphamide** (CPA) employs its metabolite mustard phosphoramide to generate alkyl bonds, hindering DNA functioning. Resultantly, this prevents the proliferation of cancerous cells. A notable phase II trial indicated its efficacy in enhancing progression-free survival especially in cases resistant to TMZ (Chamberlain & Tsao-Wei, 2004).
- **Paclitaxel trevatide**, a paclitaxel-peptide drug conjugate (PDC) utilizing the LRP-1 pathway to penetrate the blood-brain barrier, has successfully undergone phase II trials for certain cancer types and has received FDA orphan drug and fast track designations for glioblastoma multiforme treatment on May 2014 (https://angiochem.com/angiochem%E2%80%99s-ang1005-received-orphan-drug-designation-fda-treatment-glioblastoma-multiform). Developed by Angiochem, it utilizes a cleavable succinyl ester linkage to attach three paclitaxel molecules to the Angiopep-2 peptide vector and is slated for phase III trials comparing its efficacy in extending survival in specific breast cancer scenarios (Régina et al., 2008).

1.3.5.3 Radiation therapy

Radiotherapy is a commonly administered treatment to cancer patients, with approximately half of patients undergoing the therapy. This treatment utilizes ionizing radiation to severely damage or kill the DNA of cancer cells, which ultimately leads to their demise (Baskar et al., 2012). Healthy cells, on the other hand, can recover from radiation-induced damage. The main objective of radiotherapy is to spare healthy cells while targeting cancer cells. When administered preoperatively, radiotherapy aims to shrink the tumor, whereas its post-operative role is to eradicate any residual cancerous cells (Begg et al., 2011).

For GBM patients under 70 years, concurrent TMZ and radiotherapy are usually prescribed. Early initiation of treatment is emphasized to achieve optimal results. External beam radiation, following surgical resection, typically involves a 60 Gy dose delivered in two Gy fractions over 6 weeks (Sulman et al., 2017). However, for elderly patients (above 70 years), hypofractionated radiotherapy is advised. This approach

curtails tumor regrowth, accentuates cell death, and reduces therapy duration (Budach et al., 1997; Hingorani et al., 2012).

One approach suggests a 60 Gy dose in 20 fractions, producing a 40% survival rate at one year. This contrasts with the traditionally used 60 Gy dose delivered in two Gy fractions over 6 weeks, which may not be suitable for elderly patients. The use of hypofractionated radiotherapy in this age group has been shown to be effective in terms of both survival rate and reducing therapy duration. It is important to note that each patient's treatment plan should be tailored to their specific needs and circumstances, and further research is needed to determine the optimal dose and fractionation for this population (Sultanem et al., 2001).

1.4 Cancer stem cells

Stem cells, irrespective of their origin, possess the fundamental attributes of self-renewal, cloning capabilities, and the potential to differentiate into a myriad of cell types. Primarily found during embryonic, fetal, and adult phases of human development, these cells also contribute significantly to tissue repair post injury in mature organisms (Conley et al., 2005; Vats et al., 2005).

Cancer stem cells (CSCs) are unique subpopulations within malignant tumors, discovered in diverse cancers such as leukemia, breast, and brain cancers (Lapidot et al., 1994; Al-Hajj et al., 2003; Singh et al., 2003). These cells are recognized for their resilience against conventional therapies, leading to chemoresistance and recurrence. Intrinsically resembling stem cells, CSCs also harbor self-renewal capabilities (Clarke et al., 2006).

Crucial to CSC survival and functionality is their residing microenvironment or 'niches', which are regions of epithelial tissues or other systems where they remain until differentiation (Voog & Jones, 2010; Chen et al., 2013; Mohyeldin et al., 2010). Two core models, the hierarchical and stochastic, attempt to elucidate CSC formation and associated heterogeneity (Kreso & Dick, 2014; F Quail et al., 2012). Phenotypic plasticity ties these models, permitting mutual transformations between CSCs and regular cancer cells, a significant contributor to cancer persistence (Chaffer & Weinberg, 2015). (Figure 1.5)

1.4.1 Glioblastoma stem cells

Glioblastoma stem cells (GBM CSCs), a subset of CSCs, are situated in malignant gliomas and have the capability to evolve into astrocytes, oligodendrocytes, and neurons (Huang et al., 2010). Their origin remains ambiguous, but microenvironmental factors like nutrient scarcity and hypoxia can instigate their emergence (Heddleston et al., 2009; Heddleston et al., 2010).

1.4.1.1 Characteristics and genotypic aspects

The hallmarks of GBM CSCs comprise of prolific self-renewal, pluripotency, neurosphere formulation, and a pronounced mortality rate (Huang et al., 2010; Cruceru et al., 2013). Their invasive aptitude surpasses non-CSC tumors (Cheng et al., 2011). Interestingly, hypoxia plays a significant role in GBM CSC sustenance, as low oxygen zones enhance undifferentiated cell maintenance, instigate angiogenesis, and result in a more belligerent tumor phenotype (Masson, & Ratcliffe, 2014; Heddleston et al., 2009).

Genotypically, GBM CSCs exhibit certain overexpressed genes that serve as distinctive markers. Among these, prominin1 (CD133) is frequently utilized, although its functional role remains elusive (Brescia et al., 2013). Other transcription factors, including SOX2 and NANOG, are also prevalent, associating with stem cell maintenance and malignant progression, respectively (Kamachi & Kondoh, 2013; Gangemi et al., 2009; Ye et al., 2021).

1.4.1.2 Phenotypic diversity and resistance mechanisms

GBM CSC phenotypic presentations are multifarious, attributed to cellular plasticity, varying environmental factors, and genetic alterations (Gimple et al., 2019). Their resistance to conventional treatments such as temozolomide (TMZ) is often linked to the overexpression of CD133, which triggers enhanced DNA repair mechanisms (Tamura et al., 2010; Liu et al., 2006; Nakada et al., 2012). Additionally, GBM CSCs deploy overexpressed anti-apoptotic genes and ATP-binding cassette (ABC) transporters, granting them robust defense against various therapeutic drugs (Hsieh et al., 2011; Uribe et al., 2017). (Figure 1.5)

1.4.1.3 Techniques for in-depth analysis

Investigating GBM CSCs requires innovative approaches. In vitro methods such as neurosphere cultivation from GBM cell lines have gained traction due to their mimicry of three-dimensional tumor structure (Lee

et al., 2006; Wan et al., 2010). Alternatively, flow cytometry provides single-cell analysis, facilitating the detection of specific CSC markers (Ishiguro et al., 2017). However, these methodologies do come with limitations, particularly in fully representing the tumor's complexity and intricate microenvironment (Pastrana et al., 2011; Lathia et al., 2011).

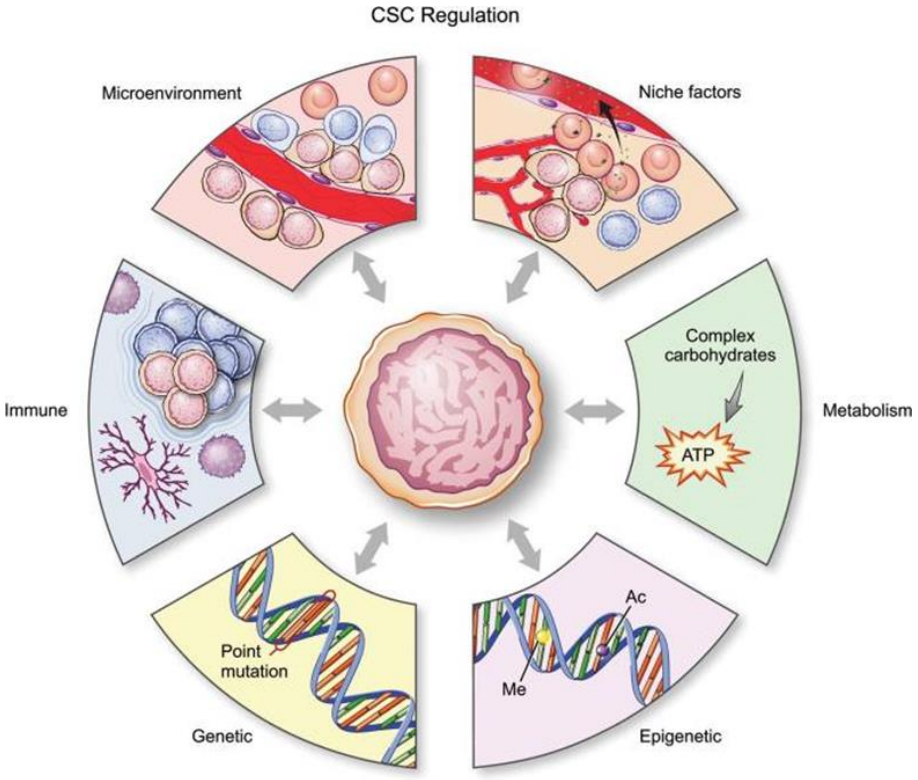

Figure 1.5: Regulation of CSCs. Cell-autonomous (intrinsic) and external (extrinsic) forces regulate the CSC state. Key intrinsic regulators include genetic, epigenetic, and metabolic regulation, while extrinsic regulators include interaction with the microenvironment, including niche factors and the immune system (Lathia et al., 2015).

1.5 Metabolic reprogramming

The investigation of metabolism in connection with cancer has been thoroughly examined, with special attention given to the capacity of tumor cells to reconfigure and reorganize metabolism to achieve superior growth, survival, and invasion abilities. This modification of metabolism has therefore been identified as one of the novel hallmarks of cancer, as noted by Fouad and Aanei (2017) and Hanahan and Weinberg (2000).

Metabolic reprogramming in cancer cells is a complex adaptive mechanism that bolsters various key features of tumorigenesis, such as rapid proliferation, survival, invasion, metastasis, and treatment resistance (Hanahan & Weinberg, 2011). The recalibration of energy metabolism has emerged as a salient component of oncogenic adaptation. This is primarily due to its pivotal role in ensuring cell survival, especially during conditions like hypoxia and cellular overgrowth where the oxygen and nutrient demands often exceed the supply (Mohyeldin et al., 2010). As tumors expand, they frequently outstrip their supply of oxygen and essential nutrients. Consequently, they engage in the process of angiogenesis, where new blood vessels are formed to fulfill the burgeoning metabolic demands (Carmeliet & Jain, 2011). This adaptative response is emblematic of metabolic reprogramming. Interestingly, anti-angiogenesis treatments have been designed to target this very adaptive strategy. Initially, these treatments can halt tumor progression by curtailing its vasculature. However, such interventions often have a counterproductive effect. The resultant oxygen and nutrient deprivation can inadvertently precipitate the emergence of treatment-resistant phenotypes in the tumor (Soda, 2013). The enduring viability of cancer cells under hypoxic conditions can be attributed to sustained metabolic reprogramming adaptations, such as the Warburg effect, where cancer cells predominantly produce energy through glycolysis despite the presence of oxygen (Warburg et al., 1931).

1.5.1 Warburg effect

The Warburg effect, coined after Otto Warburg's pioneering work in 1927, delineates a unique metabolic adaptation of cancer cells where they predominantly utilize aerobic glycolysis. Even in oxygen-rich conditions, these cells exhibit heightened glucose uptake and conversion to lactate (Warburg, Wind, & Negelein, 1927). This phenomenon elucidates how cancer cells exploit metabolic flexibility to ensure their sustenance and proliferation. It's suggested that such a metabolic shift caters to the elevated energy demands of these cells, aiding in efficient nutrient assimilation into biomass (Vander Heiden, Cantley, & Thompson, 2009). However, the exact reason for this transition from oxidative phosphorylation to aerobic glycolysis in certain cancer cells remains enigmatic, and contemporary studies indicate that oxidative phosphorylation could also spur cancer growth (DeBerardinis & Chandel, 2020). Recent studies have highlighted the role of oxygen in enhancing lactate synthesis, both from glucose and glutaminolysis, as a key factor in the Warburg effect (Graboń, 2018). This lactate production, termed "lactagenesis," is proposed to support carcinogenesis and drive the Warburg effect (San-Millán, 2016). The Warburg effect is also influenced by various regulatory mechanisms, including mutations in oncogenes and tumor suppressor genes (Bensinger, 2012). With metabolic reprogramming being fundamental to cancer

progression, it is now acknowledged as one of cancer's hallmarks, painting cancer as predominantly a metabolic disease (Hanahan & Weinberg, 2011; Hanahan, 2022). Notably, while normal cells resort to glycolysis under hypoxic conditions and subsequently process pyruvate in the mitochondria, cancer cells, owing to the Warburg effect, rely heavily on glycolysis for energy production even in oxygen's presence (Wang & Dong, 2019; Warburg, 1956). Anaplerosis, the process of replenishing TCA cycle intermediates, is crucial for maintaining cellular metabolism. Pyruvate carboxylation, a key anaplerotic reaction, plays a significant role in this process (Wattanavanitchakorn, 2019). The Warburg effect, characterized by increased glycolysis and lactate production, is a hallmark of cancer cells. This metabolic shift can lead to an increased demand for anaplerotic reactions, including pyruvate carboxylation, to maintain TCA cycle intermediates (Crociani, 2018). The regulation of pyruvate metabolism, including the activity of pyruvate carboxylase, is a potential target for therapeutic interventions in metabolic-related diseases (Jeoung 2013). Plasticity could be strategically targeted for therapeutic interventions (Lee, 2014). (Figure 1.6)

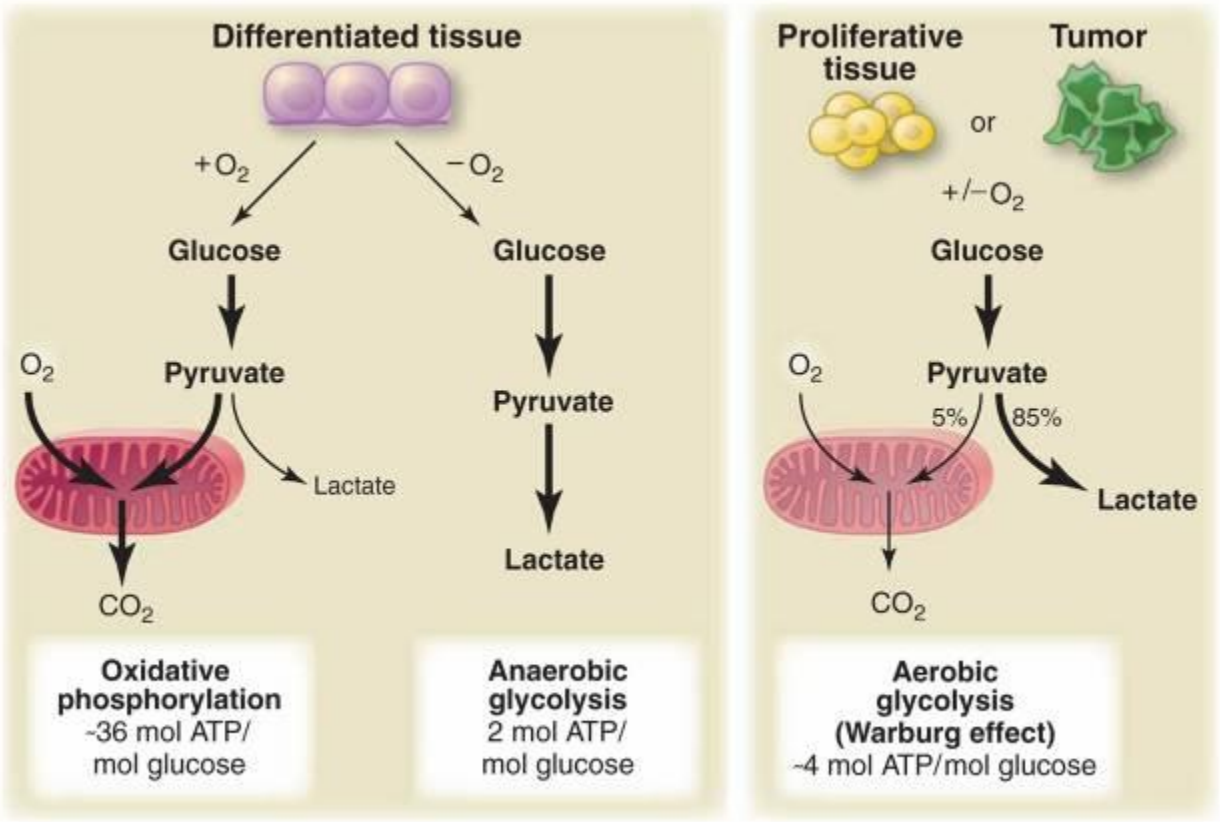

Figure 1.6: Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (Warburg effect) (Vander Heiden, Cantley, & Thompson, 2009).

1.5.2 Glucose-6-phosphatase

No matter which method tumors choose for energy metabolization or what reprogrammed pathway it uses, a primary substance, in this case, glucose, is always needed. One of the most significant enzymatic systems contributing to glucose homeostasis and glycogenolysis is the glucose-6-phosphatase system located at the endoplasmic reticulum membrane (Chou et al., 2010). The glucose-6-phosphatase catalytic subunit (G6PC) and the glucose-6- phosphate translocase (G6PT/SLC37A4) are the major components which formed this complex partnering to transport G6P to the ER lumen from the cytoplasm (G6PT) and to hydrolyze G6P (G6PC) (Khan et al., 2015). Studies show that the expression of G6Pase is downregulated in cancers like HCC and RCC with gluconeogenic tissue, while in non-gluconeogenic tissue tumors such as glioblastoma and ovarian cancer; it is upregulated (Guo et al., 2015). As mentioned above, the role of G6PC in glycogenolysis both helps the tumor with survival and migration under stress and builds resistance in the face of 2-deoxyglucose treatment (Guo et al., 2015). It was also established that silencing G6PC in ovarian cancer leads to accumulation of glycogen, down-regulation of PYGL (regulator in glycogenolysis), reduction in cell growth, and suppression of Epithelial-Mesenchymal Transition (EMT) (Hutton & O'Brien, 2009).

These findings try to answer the question of how the G6PC and SLC37A families are related to cancer survival and phenotype and are a start of how to target them for therapeutic matters.

1.5.2.1 G6PC family

G6PC1, G6PC2, and G6PC3 are members of the G6PC family. Despite the differences in their expression patterns, amino acids, related diseases, and functionality, all three members of the G6PC family can hydrolyze G6P. The rate of hydrolyzing activity of G6PC2 and G6PC3 (G6PCβ) is, however, much lower than G6PC1 (G6PCα) (Mansfield, 2014). **G6PC1**, an enzyme that catalyzes the final step in gluconeogenic and glycogenolytic pathways, plays an essential role in maintaining euglycemia, particularly in the fasted state, and has been identified to have implications in various diseases and metabolic conditions when mutated or overexpressed (Chou & Mansfield, 2008). Notable mutations in the G6PC1 gene induce Glycogen Storage Disease (GSD) type 1a, manifesting in severe hypoglycemia and other metabolic disruptions, while overexpression is linked with disturbances in glucose metabolism, notably within the contexts of type 1 and type 2 diabetes (O'Brien et al., 2001). Regulatory factors and transcriptional controls of G6PC gene expression have been explored, revealing the intricate network of hormonal and metabolic agents, such as glucagon and insulin, which modulate its activity. Additionally, the enzyme's transcriptional regulation

in various tissues, predominantly the liver, has been scrutinized, shedding light on the multifaceted interactions involving several hormones and metabolites that govern G6PC1 expression and, by extension, hepatic glucose production (Hutton & O'Brien, 2009). Various co-activators and their impact on G6PC1 expression have also been studied, offering insights into the complex regulatory networks that oversee glucose homeostasis in physiological and pathophysiological states (Hutton & O'Brien, 2009).

G6PC2, an enzyme with considerable amino acid identity to G6PC, has witnessed debate concerning its ability to hydrolyze glucose-6-phosphate (G6P) with variable experimental results. Examination of G6pc2 null mice indicated its potential involvement in modulating blood glucose levels post-fasting and influencing glucose-stimulated insulin secretion (GSIS) in vivo (Martin et al., 2001). While G6PC2 has been implicated in type 1 diabetes pathophysiology and cell-mediated autoimmunity, its nuanced role still warrants further exploration. Notably, a genome-wide association study correlated G6PC2 gene SNPs with variations in human fasting blood glucose levels, potentially impacting cardiovascular-associated mortality due to even minor fluctuations in fasting glucose levels. Future research is required to decipher the precise SNP linking G6PC2 expression to blood glucose modulation (Wang et al., 2007).

G6PC3, sharing approximately 36% amino acid identity with G6PC, has presented inconclusive data regarding its capacity to hydrolyze G6P, with various experimental approaches yielding different results (Wang et al., 2006). Investigations utilizing G6pc3 null mice revealed a decrease in G6P hydrolytic activity, particularly noted in brain and testis tissues, and distinct physiological consequences, including gender-specific growth retardation and varied plasma glucagon and cholesterol levels, suggesting a complex in vivo function (Shieh et al., 2004). Furthermore, G6PC3 mutations have been associated with a severe congenital neutropenia syndrome, alongside cardiac and urogenital malformations, while its transcriptional regulation remains largely unexplored, highlighting the G6PC3 promoter's distinctive lack of a TATA box and uncharacterized promoter elements (Hutton & O'Brien, 2009).

1.5.2.2 SLC37A family

Glucose-6-phosphate transporter (G6PT) or SLC37A4 is the most known out of the four members of the SLC37A family. Apart from SLC37A3, three other members have roles in the terminal step of the gluconeogenic and glycogenolytic pathways. However, unlike SLC37A4 functionally coupled with G6PCα and G6PCβ to hydrolyze G6P, SLC37A1 and SLC37A2 cannot couple with these two proteins to function.

Unfortunately, the biological role of SLC37A1, SLC37A2, and SLC37A3 has not been determined yet (Mansfield, 2014).

G6PT, encoded by the **SLC37A4** gene, plays a critical role in translocating glucose-6-phosphate (G6P) from the cytoplasm into the endoplasmic reticulum (ER) lumen, where G6P is hydrolyzed by either G6Pase-α or G6Pase-β (Chou et al., 2002). Notably, G6PT and its variant vG6PT, despite having different expression patterns across tissues, exhibit equal efficacy in G6P uptake. G6PT not only facilitates the transport of cytoplasmic G6P into the ER lumen but also exhibits a physical interaction that augments G6P transport activity, presumably through an allosteric mechanism (Chou et al., 2010). This G6PT-G6Pase functional coupling has been demonstrated through various methodologies, including hepatic microsomal G6P uptake activity in GSD-Ia mice and cell-based activity assays for recombinant G6PT proteins (Zhu et al., 2022). Additionally, G6PT has been revealed to be a Pi-linked G6P antiporter, catalyzing G6P:Pi and Pi:Pi exchanges in a model using reconstituted proteoliposomes. Investigations of SLC37A4 mutations in Glycogen Storage Disease type Ib (GSD-Ib) patients indicate significant disparities in G6P and Pi transport activities, hinting at potential mechanistic insights into this genetic disorder (Chen et al., 2008). Moreover, mutation p.Q133P retains minimal wild-type Pi transport activity, yet is devoid of G6P transport activity, highlighting the possible divergence of active sites for G6P and Pi transport. Intriguingly, despite earlier biochemical classifications suggesting GSD-Ic as a distinct subtype characterized by a lack of Pi transport activity, recent genotyping has uncovered deleterious SLC37A4 mutations in GSD-Ic patients, aligning with G6PT's dual role as both a G6P and Pi transporter, thus uniting previously segmented understanding and emphasizing the complexity and necessity of further investigating G6PT and associated pathologies (Chen et al., 2008).

The **SLC37A1** gene, located on chromosome 21q22.3, encodes a polypeptide and shows homology to several proteins within its family and a 30% sequence identity with GlpT, suggesting a possible role as a glycerol-3-phosphate transporter. While it's broadly expressed in several organs, SLC37A1 demonstrates varied transcript levels compared to SLC37A4 across different tissues and is notably upregulated by the epidermal growth factor in certain cancer cells (Bartoloni et al., 2000). Despite hypothesized involvement in phospholipid biosynthesis and tumor cell proliferation, a direct role in diseases, including cancer, has not been empirically substantiated. Neutrophils, displaying higher SLC37A1 transcript levels compared to SLC37A4, pose as a viable cellular model for exploring its biological function (Chou et al., 2013).

SLC37A2, encoded by 18 exons on chromosome 11q24.2, exhibits notable homology with other SLC37 family members and distinctively undergoes N-linked glycosylation, unlike its paralogs. Originally identified as a cAMP-inducible gene in murine macrophages, it expresses predominantly in spleen, thymus, and macrophages, with substantial expression in neutrophils. Its post-translational modification and heightened transcript levels during THP-1 monocyte-to-macrophage differentiation suggest a potential biological transport role within macrophages and neutrophils (Chou & Mansfield, 2014). Comprising 17 exons on chromosome 7q34, **SLC37A3** produces three alternative transcripts with expression identified in various organs and cell types, notably with elevated levels in pancreas and neutrophils compared to its family counterparts. The conspicuous expression of SLC37A3 and its homologs in neutrophils invites further investigation into their collective role in immunological processes and underscores the potential undiscovered functionalities of SLC37A3 in the pancreas and immune cells (Chou & Mansfield, 2014).

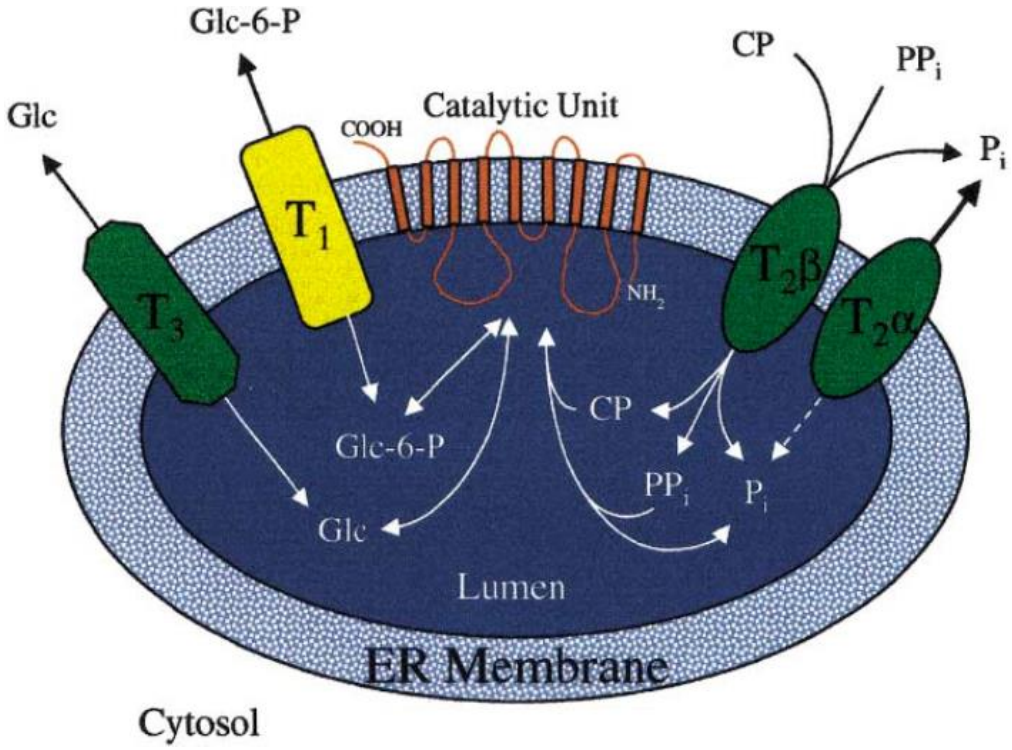

Figure 1.7: The glucose-6-phosphatase system. Structure-function relationship of the G6Pase system acCording to the substrate translocase-catalytic unit hypothesis. A cross-section of the ER is depicted: T1 (G6PT). T2lX. T213. and T3 are sUbstrate/product transporters and/or auxiliary proteins with the indicated specificity; Catalytic Unit is G6Pase (EC 3.1.3.9) embedded Withinthe ER membrane with nine-transmembrane-spanning helical regions. (Wallert et al., 2001).

1.6 Research project

1.6.1 Hypothesis

Recent advances in glioblastoma multiforme (GBM) metabolism highlight the pivotal role of the glucose-6-phosphatase (G6Pase) system in the pathophysiology of this aggressive brain cancer. It is hypothesized that the gene expression levels of the G6Pase system components, specifically G6PC and SLC37A family members, are significantly altered in GBM tissues. These alterations are postulated to play a critical role in regulating and sustaining the invasive characteristics and the phenotype of brain cancer stem cells (CSCs), which are key contributors to GBM progression and therapeutic resistance. The aberrant expression of these genes is believed to facilitate the metabolic reprogramming of GBM cells, aiding their survival and proliferation within the hypoxic tumor microenvironment. Consequently, these molecular changes may support GBM cells' adaptability and resistance to conventional therapies, thus impacting the disease's prognosis. This hypothesis will be tested by examining and analyzing the gene expression patterns and their functional implications in GBM, with an emphasis on the roles of G6PC and SLC37A family members in the metabolic adaptation and stemness properties of GBM cells.

1.6.2 Objectives

The study aimed to provide a detailed understanding of the regulation and functional significance of G6PC and SLC37A members in the context of glioma progression and CSC phenotype acquisition. The current study aimed to investigate the objectives by conducting a comprehensive analysis at the transcriptional level. The primary focus was to determine the extent to which the expression of G6PC and SLC37A members were regulated in various scenarios such as in different cell lines. Firstly, the investigation explored the transition from a healthy brain to low-grade glioma, followed by the progression to GBM tissues. Additionally, the study also examined the regulation of G6PC and SLC37A members in established human GBM cell line models. Furthermore, the contribution of these members in the chemotactic response and the acquisition of a cancer stem cell (CSC) phenotype were thoroughly explored.

CHAPITRE 2

Article

A molecular signature for the G6PC3 / SLC37A2 / SLC37A4 interactors in glioblastoma disease progression and in the acquisition of a brain cancer stem cell phenotype

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Authors' contributions

Sima Torabidastgerdooei: Experimentation, data analysis, figure assembly, manuscript writing

Borhane Annabi: Conceptualization of experiments, financial support, data analysis, drafting of manuscript

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A molecular signature for the G6PC3 / SLC37A2 / SLC37A4 interactors in glioblastoma disease progression and in the acquisition of a brain cancer stem cell phenotype

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Running title: Metabolic reprogramming of brain cancer cells

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Keywords: Glioblastoma, Glucose-6-phosphatase system, G6PC3, G6PT, SLC37A2, SLC37A4, Cancer stem cells, Metabolic reprogramming

2.1 Abstract

Background: Glycogen plays an important role in glucose homeostasis and contributes to key functions related to brain cancer cell survival in glioblastoma multiforme (GBM) disease progression. Such adaptive molecular mechanism is dependent on the glycogenolytic pathway and intracellular glucose-6-phosphate (G6P) sensing by brain cancer cells residing within those highly hypoxic tumors. The involvement of components of the glucose-6-phosphatase (G6Pase) system remains, however, elusive. **Objective**: We questioned the gene expression levels of components of the G6Pase system in GBM tissues and their functional impact in the control of the invasive and brain cancer stem cells (CSC) phenotypes. **Methods**: In silico analysis of transcript levels in GBM tumor tissues was done by GEPIA. Total RNA was extracted and gene expression of G6PC1-3 as well as of SLC37A1-4 members analyzed by qPCR in four human brain cancer cell lines and from clinically annotated brain tumor cDNA arrays. Transient siRNA-mediated gene silencing was used to assess the impact of TGF-β-induced epithelial-to-mesenchymal transition (EMT) and cell chemotaxis. Three-dimensional (3D) neurosphere cultures were generated to recapitulate the brain CSC phenotype. **Results**: Higher expression in G6PC3, SLC37A2, and SLC37A4 was found in GBM tumor tissues in comparison to low-grade glioma and healthy tissue. The expression of these genes was also found elevated in established human U87, U251, U118, and U138 GBM cell models compared to human HepG2 hepatoma cells. SLC37A4/G6PC3, but not SLC37A2, levels were induced in 3D CD133/SOX2-positive U87 neurospheres when compared to 2D monolayers. Silencing of SLC37A4/G6PC3 altered TGF-β-induced EMT biomarker SNAIL and cell chemotaxis. **Conclusion**: Two members of the G6Pase system, G6PC3 and SLC37A4, associate with GBM disease progression and regulate the metabolic reprogramming of an invasive and CSC phenotype. Such molecular signatures may support their role in cancer cell survival and chemoresistance and become future therapeutic targets.

2.2 Introduction

The metabolic reprogramming of cancer cells is a key contributing factor to tumorigenesis enabling rapid proliferation, survival, invasion, metastasis, resistance to treatments, and other processes associated with carcinogenesis (Navarro et al. 2022, Ward & Thompson 2012, Nong et al. 2023). Reprogramming energy metabolism is also an important adaptive mechanism exploited by hypoxic solid tumors to increase cell survival within a low oxygen tumor microenvironment (Belisario et al. 2020, Schiliro & Firestein 2021, Liberti & Locasale 2016). As such, hypoxic cancer cells exhibit the Warburg effect allowing them to use glycolysis to convert glucose into pyruvate as their primary source of energy production, even in the presence of oxygen (aerobic glycolysis) (Bose et al. 2021, Liu et al. 2021). In cases of nutrient deficiency,

such as lack of glucose, this method of energy production may not be efficient. Thus, as cancer cells constantly activate additional metabolic reprogramming to complete their energy needs, a better understanding of glucose metabolism and enzymatic systems involved will help design better cancer therapeutic strategies (Lin et al. 2020).

Of particular interest, the flux in glucose/glucose-6-phosphate (G6P) can be sensed, in part, through components system of the glycogen metabolism, which appear dysregulated in a wide variety of malignancies (Khan et al. 2020). Indeed, the levels of glycogen were demonstrated to be particularly high in breast, kidney, uterus, bladder, ovary, skin, and brain cancer cell lines (Zois et al. 2014). The glucose-6-phosphatase (G6Pase) system, located at the endoplasmic reticulum (ER) membrane, has been identified as a significant enzymatic system in the regulation of glucose homeostasis and in glycogenolysis (van Schaftingen & Gerin 2002, Chou et al. 2002), and deficiencies responsible for type I glycogen storage disease (Chou et al. 2015). It is composed of two main components, the G6P translocase (G6PT/SLC37A4) and the G6Pase catalytic subunit (G6PC). SLC37A4 senses and transports G6P from the cytoplasm to the ER lumen where it is hydrolyzed by the G6PC (Cappello et al. 2018, Chou & Mansfield 2014), the latter activity being linked to glycogen turnover in cancer cells (Grasmann et al. 2019). In hepatocellular carcinoma (HCC), G6PC expression (Wang et al. 2012, Ma et al. 2013, Khan et al. 2015) and activity (Wang et al. 2012) were downregulated compared with adjacent tumor-free tissues, and similar results were obtained in renal cell carcinoma (RCC) (Khan et al. 2015, Li et al. 2014). In contrast, G6PC expression was enhanced in glioblastoma (GBM), a highly malignant brain cancer, compared to the noncancerous human cortex (Abbadi et al. 2014). Tumor-initiating cells isolated from GBM showed an upregulation of G6PC by the glycolysis inhibitor 2-desoxyglucose (2DG) (Abbadi et al. 2014). G6PC silencing reduced proliferation and migration of GBM cells and invasion in vivo, which was especially pronounced after 2DG treatment and recovery. Mechanistically, G6PC silencing is believed to lead to glycogen accumulation and to result in reduced activation of AKT (protein kinase B) (Abbadi et al. 2014).

High G6PC expression was found to be associated with poor overall and disease-free survival in ovarian cancer (Guo et al. 2015). Here again, silencing G6PC in ovarian cancer was associated with an accumulation of glycogen, a downregulation of PYGL (a regulator of glycogenolysis), a reduction in cell growth, and in an inhibition of the epithelial-to-mesenchymal transition (EMT) (Guo et al. 2015). Interestingly, glycogen accumulation is also a key initiating oncogenic event during liver malignant transformation where G6PC is frequently downregulated to augment glucose storage in pre-malignant cells (Liu et al. 2021). Consistently,

the elimination of glycogen accumulation abrogated liver growth and cancer incidence, whereas increasing glycogen storage appeared to accelerate tumorigenesis. It is further hypothesized that cancer-initiating cells adopt a glycogen storing mode to augment tumor incidence.

The G6PC family consists of three members: G6PC1 (G6PCα), G6PC2, and G6PC3 (G6PCβ) (Hutton & O'Brien 2009). Despite their distinct expression patterns and associated diseases, all three members can hydrolyze G6P (Marcolongo et al. 2013), although the hydrolyzing activity of G6PC2 and G6PC3 is believed to be much lower than that of G6PC1 (Hutton & O'Brien 2009). In addition, the SLC37A family members are ER-associated sugar-phosphate/phosphate (P(i)) exchangers consisting of four members: SLC37A1, SLC37A2, SLC37A3, and SLC37A4. Aside from SLC37A3 which function is unknown, the other three members play roles in the final step of the gluconeogenic and glycogenolytic pathways (Chou & Mansfield 2014). However, unlike SLC37A4, which is functionally coupled with G6PCα and G6PCβ to hydrolyze G6P, SLC37A1 and SLC37A2 cannot couple with these two proteins to function (Chou, Sik Jun & Mansfield 2013).

Several underestimated roles for the SLC37A4 as a potential regulator of human U87 GBM cancer cells' invasive phenotype and of angiogenic processes were documented in brain endothelial cells (Belkaid et al. 2006, Tahanian et al. 2010). In addition, increased SLC37A4 transcriptional regulation under hypoxic culture conditions was also reported to require hypoxia inducible factor (HIF)-1α (Lord-Dufour et al. 2009). SLC37A4's potential role included regulation of calcium-mediated signaling, which is known to control cancer cell proliferation, cell cycle division, extracellular matrix degradation, and response to growth factors (Fortier et al. 2008, Belkaid et al. 2007).

While GBM is a highly aggressive form of brain cancer (Seker-Polat et al. 2022), recent advances in our understanding of their metabolism suggests that it is highly heterogeneous, and that cancer stem cells (CSC), a small subset of all cancer cells, may further exhibit specific metabolic traits that could play a significant role in anticancer therapy failure (Bernhard et al. 2023). The objectives of the current study were to address, as a first investigatory step at the transcriptional level, to what extent the expression of G6PC and SLC37A members were regulated in i) the transition from healthy brain to low-grade glioma, then to GBM tissues, and ii) in established human GBM cell line models. Moreover, their contribution in iii) the chemotactic response and the acquisition of a CSC phenotype were also explored. Understanding CSC biology and metabolic reprogramming involving the G6Pase components could become keys to optimizing anticancer treatments (Gupta, Chaffer & Weinberg 2009).

2.3 Materials and Method

2.3.1 Materials

Sodium dodecyl sulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Canada (Oakville, ON). Cell culture media was obtained from Life Technologies (Burlington, ON). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). All other reagents were from Sigma-Aldrich Canada.

2.3.2 In silico analysis of transcripts levels in clinical glioblastoma and low-grade glioma tissues

A Gene Expression Profiling Interactive Analysis (GEPIA) web server was used to analyze the RNA sequencing expression data of glioblastoma tumors (GBM, n = 163) vs. healthy tissue (n = 207), and of low-grade glioma (LGG, n = 251) vs. healthy tissue (n = 207) from the TCGA and the normal brain tissue in Genotype-Tissue Expression (GTEx) databases (Tang et al. 2019). GEPIA provides customizable functions such as tumor/normal differential expression analysis, profiling according to cancer types or pathological stages, patient survival analysis, similar gene detection, correlation analysis, and dimensionality reduction analysis (http://gepia.cancer-pku.cn/detail.php, accessed on July 5th, 2023). One-way ANOVA was used for differential analysis of gene expression, using disease states (GBM, LGG, or normal) as variables for the box plots.

2.3.3 Prognostic value of G6PC3, SLC37A2, and SLC37A4 in glioblastoma patients

The prognostic value of mRNA level of G6PC3, SLC37A2, and SLC37A4 factors in GBM patients was analyzed by GEPIA (Tang et al. 2019). For each of the three genes tested, browsing of human gene-expression fingerprints was retrieved from a web-based database containing a large number of high-quality data sets in GBM tissues. Log-rank tests for overall survival analyses were used.

2.3.4 Protein-protein interaction

The associative relationships of G6PC3 and of SLC37A2 were retrieved from the STRING v11 database (https://www.string-db.org/) to identify and build protein-protein interaction networks (Szklarczyk et al. 2021), with a confidence score setting of 0.4, and the maximum number of interactions to show was no more than 10.

2.3.5 Cell culture

The human U87 (HTB-14), U118, U138, and U251 glioblastoma cell lines, as well as the human HepG2 hepatoma cell line, were from American Type Culture Collection (Manassas, VA). They were all maintained in Eagle's Minimum Essential Medium (Wisent, 320-006CL) containing 10% (v/v) calf serum (HyClone Laboratories, SH30541.03), 2 mM glutamine, 1 mM sodium pyruvate (Sigma-Aldrich Canada, P2256), 100 units/ml penicillin and 100 mg/ml streptomycin (Wisent, 250-202-EL). Cells were incubated at 37°C with 95% air and 5% CO2. Neurosphere formation was performed as follows: 80-90% adherent U87 monolayer cells were trypsinized and plated in low adhesion 24-well plates (Corning Costar, Corning, NY, USA) at a density of 2x105 cells/mL in complete media for 24-72 hours. Then, the supernatant was removed, and serum-free EMEM supplemented with 10 ng/mL human basic fibroblast growth factor (Gibco, Thermo Fisher, 13256029), 20 ng/mL human epidermal growth factor (Gibco, Thermo Fisher, PHG0315), 5 µg/mL insulin (Sigma Aldrich Corp, I3536,) and BSA (Sigma Aldrich Corp, A9418-5G,) at 4% was carefully added to the dishes. Spheroids were defined as rounded aggregates of cells with a smooth surface and poor cell-to-cell definition. Perimeters of 30-70 spheroids/flask were assessed for each experimental condition performed in triplicate and derived from three independent experiments.

2.3.6 TissueScan cDNA arrays of grades I-IV brain tumor tissues

TissueScanTM cancer and normal tissue cDNA arrays were purchased from OriGene (Rockville, MD), covering 43 clinical samples of the four stages of brain cancer as well as normal tissues, and were used to assess G6PC3, SLC37A2, and SLC37A4 gene expression levels according to the manufacturer's recommendations. Tissue cDNAs in each array were synthesized from high-quality total RNAs of pathologist-verified tissues, normalized and validated with ß-actin in two sequential qPCR analyses, and accompanied by clinical information for 18 WHO grade I, 11 WHO grade II, 10 WHO grade III, and 2 WHO grade IV brain tumors.

2.3.7 Total RNA isolation, cDNA synthesis and real-time quantitative RT-PCR

Total RNA was extracted from cell monolayers using TriZol reagent (Life Technologies, 15596-018). For cDNA synthesis, 2 µg of total RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, 4368814). cDNA was stored at -80°C prior to PCR. Gene expression was quantified by real-time quantitative PCR using Sso Fast EvaGreen Supermix (Bio-Rad). DNA amplification was carried out using a CFX Connect Real-Time System (Bio-Rad) and product detection was performed by measuring the binding of the fluorescent dye EvaGreen to double-stranded DNA. The following QuantiTect primer

sets were provided by QIAGEN : G6PC1 (Hs_G6PC_1_SG QT00031913), G6PC2 (Hs_G6PC2_va.1_SG QT01664152), G6PC3 (Hs_G6PC3_1_SG QT00033453), SLC37A1 (SLC37A1_1_SG QT00073094), SLC37A2 (Hs_SLC37A2_1_SG QT00056203), SLC37A3 (Hs_SLC37A3_1_SG QT00057148), SLC37A4 (Hs_SLC37A4_1_SG QT00024325), GAPDH (Hs_GAPDH_2_SG QT01192646), β-actin (Hs_Actb_2_SG QT01680476) and PPIA (Hs_PPIA_4_SG QT01866137). The relative quantities of target gene mRNA compared against two internal controls chosen from GAPDH, β-actin or PPIA RNA, were measured by following a ΔCT method employing an amplification plot (fluorescence signal vs. cycle number). The difference (ΔCT) between the mean values in the triplicate samples of the target gene and those of GAPDH and β-actin mRNAs were calculated by CFX manager Software version 2.1 (Bio-Rad) and the relative quantified value (RQV) was expressed as $2^{-\Delta CT}$.

2.3.8 Transfection method and RNA interference

For gene silencing experiments, U87 glioblastoma cells were transiently transfected with siRNA sequences using Lipofectamine-2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA). Gene silencing was performed using 20 nM siRNA against G6PC3 (Hs_G6PC3_5 siRNA, SI02659363), SLC37A2 (Hs_SLC37A2_7 siRNA, SI04151840), SLC37A4 (Hs_SLC37A4_3 siRNA, SI00724213), or scrambled sequences (AllStar Negative Control siRNA, 1027281). The above small interfering RNA and mismatch siRNA were all synthesized by QIAGEN and annealed to form duplexes. Gene silencing efficacy was assessed by RT-qPCR as described above.

2.3.9 Real-time cell migration assay

Experiments were carried out using the Real-Time Cell Analyser (RTCA) Dual-Plate (DP) Instrument and the xCELLigence system (Roche Diagnostics, QC), following the instructions of the supplier. U87 cells were transfected with 2 nM siRNAs (Control and G6PC3) as described above. After transfection, 25,000 cells per well were seeded in a CIM-plate 16 (Roche Diagnostics) and incubated at 37°C under a humidified atmosphere containing 5% CO2 for 24 hours. Prior to cell seeding, the underside of each well in the upper chamber was coated with 0.15% gelatin in PBS and incubated for 1 hour at 37°C. The lower chamber was filled with serum-free medium. The upper chamber of each well was filled with 250,000 cells. After 30 min of adhesion, cell migration was monitored every 5 min for 20 hours. The impedance value was measured by the RTCA DP Instrument and expressed as an arbitrary unit called the Cell Index. Each experiment was performed in quadruplicate wells.

2.3.10 Statistical data analysis

Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired t-test or 1-way ANOVA with a Dunnett post-test. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance in the figures.

2.4 Results

2.4.1 Increased gene expression of G6PC3, SLC37A2, and SLC37A4 in clinically-annotated glioblastoma tumor tissues.

As previously mentioned in the Methods section, an *in silico* differential analysis of G6PC (G6PC1, G6PC2, G6PC3) (Figure 2.1A) and SLC37A (SLC37A1, SLC37A2, SLC37A3, SLC37A4) (Figure 2.1B) family members transcript levels were conducted on clinical samples from glioblastoma (GBM) and low-grade glioma (LGG) and compared to healthy brain tissues. The expression of G6PC1 and G6PC2 were very low in both LGG and GBM samples (Figure 2.1A, left and middle panels), whereas that of G6PC3 was high in both tissues and increased significantly when compared to healthy brain tissue (Figure 2.1A, right panel). All four SLC37A members were significantly expressed in LGG and GBM tissues (Figure 2.1B, red boxes). When compared to healthy tissues, only SLC37A2 was significantly increased in LGG and GBM samples, whereas the expression of SLC37A4 increased only in GBM samples and not in LGG (Figure 2.1B). This suggests that these three gene candidates may possibly serve as biomarkers of brain cancer disease progression. Their transcript expression was next further assessed in tumor tissues cDNA arrays using clinically annotated GBM samples from all four stages.

2.4.2 High G6PC3, SLC37A2, and SLC37A4 gene expression correlates with poor prognosis in GBM patients.

With the same online analytical tool, a survival analysis was performed of the three genes that showed increased expression of their transcript levels in tumor tissues (Figure 2.1, red box). When the analysis was performed with high expression of these three genes, the overall survival rate was reduced significantly (Figure 2.2A, red lines). Further, RT-qPCR analysis was performed using brain tissue scan arrays as described in the Methods section. The results show that compared to healthy brain tissue, as the grades of GBM increased, the expression of these three genes was also induced (Figure 2.2B).

2.4.3 Protein-protein interaction network predicts G6PC3 interrelationship with SLC37A4, but not SLC37A2.

A protein-protein interaction (PPI) network of G6PC3 was constructed by using the STRING database (https://string-db.org/) and was used to predict and analyze potential or existing PPI. Indirect target proteins of G6PC3 were retrieved from STRING as described in the Methods section and predicted G6PC3 interrelationship with potential biomarkers involved in the G6Pase system, namely G6PC1 and SLC37A4 (Figure 2.3A). Given that no relationship was found between G6PC3 and SLC37A2, an independent interaction network analysis was performed which confirmed that predicted PPI network did not involve components of the G6Pase system (Figure 2.3B).

2.4.4 Expression of G6PC3, SLC37A2, and SLC37A4 is increased in several human glioblastoma cell lines.

To further validate the *in-silico* analysis and cDNA array results, total RNA was extracted from four different human glioblastoma cell lines (U87, U251, U118, U138) and from one human hepatoma cell line (HepG2). Primers and RT-qPCR results were validated with agarose gel electrophoresis, which showed a single amplicon product for each of the genes amplified (Figure 2.4A). Next, qPCR analysis revealed that the expression of G6PC3, SLC37A2, and SLC37A4 was considerably higher in all four glioma cell lines tested when compared to HepG2 cells (Figure 2.4B).

2.4.5 G6PC3 and SLC37A4 levels are induced in CD133/SOX2-positive U87-derived neurospheres.

Neurospheres culture conditions are known to recapitulate, in part, the cancer stem cells (CSC) phenotype (Gupta, Chaffer & Weinberg 2009, Venere et al. 2011). U87 glioblastoma 3D neurosphere cultures appeared to reach maturation at 72 hours (Figure 2.5A) as described previously (Gresseau et al. 2022). To address how such phenotype impacted the G6PC3, SLC37A2, and SLC37A4 transcript levels, total RNA was isolated, and qPCR analysis was performed in comparison to 2D cell monolayers. The results revealed increased expression in G6PC3 and SLC37A4 transcripts in neurospheres as compared to adherent cells, while SLC37A2 levels remained unaltered (Figure 2.5B, left panel). In addition, the expression level of the CSC markers CD133, SOX2, and of epithelial-to-mesenchymal transition (EMT) markers FN1 and SNAIL were also increased in neurospheres compared to adherent cells (Figure 2.5B, right panel). CD133, which is neurospheres positive control, is associated with resistance to in vitro chemotherapy and therefore may relate G6PC3 (G6PCβ) and SLC37A4 (G6PT) to some chemoresistance and invasive molecular signature of GBM-derived CSC.

2.4.6 Silencing of G6PC3 and SLC37A4 alters the acquisition of a CSC phenotype and the expression of EMT-related biomarkers.

We next wished to address the potential crosstalk between the acquisition of CSC phenotype and the corresponding increase in G6PC3 and SLC37A4 expression upon neurospheres formation. U87 glioblastoma monolayer cells were transiently transfected with a scrambled sequence (siScrambled, Figure 2.6A, white bars) or siRNA directed against G6PC3 (siG6PC3, Figure 2.6A, left panel, black bars), or SLC37A4 (siSLC37A4, Figure 2.6A, right panel, black bars). Total RNA was then extracted, and the specificity of gene silencing confirmed for each repressed gene using RT-qPCR. Next, transfected U87 cells were cultured with the Tumorsphere Medium Xf with SupplementMix for 72 hours to generate neurospheres. Total RNA was again extracted from neurospheres and selected CSC (CD133, SOX2) and EMT (FN1, SNAIL) markers analyzed by RT-qPCR in siScrambled (Figure 2.6B, white bars), siG6PC3 (Figure 2.6B, black bars), and siSLC37A4 (Figure 2.6B, grey bars). In all the conditions tested, the expression of CD133, SOX2, SNAIL, and FN1 was all prevented in neurospheres upon either G6PC3 or SLC37A4 repression. This could suggest that metabolic reprogramming involving these two genes are important factors that contribute to the acquisition of a CSC phenotype and induction of EMT biomarkers during neurospheres formation possibly through their respective G6P sensing (SLC37A4) and/or hydrolyzing (G6PC3) activities.

2.4.7 Evidence for G6PC3 and SLC37A4 involvement in the chemotactic response of U87 glioblastoma cells to TGF-β.

Induced expression of EMT biomarkers SNAIL and FN1 documented above appears to play a role in the acquisition of a CSC phenotype in neurospheres. We thus questioned whether any early signaling events could be mimicked in such cellular response to transforming growth factor-beta (TGF- β), a known potent EMT inducer in glioblastoma cells (Djediai et al. 2021, Ouanouki, Lamy & Annabi 2018), and how G6PC3 and SCL37A4 would be involved. U87 monolayer cells were transiently transfected with specific siRNA to repress the expression of G6PC3, SLC37A2, and SLC37A4, or with a scrambled siRNA (Control). Cells were then challenged with 10 nM TGF- β for 24 hours. Total RNA was extracted, followed by RT-qPCR assessment of SNAIL gene expression. TGF- β effectively induced SNAIL in siScrambled-transfected cells, and this was significantly inhibited upon G6PC3 and SLC37A4 gene silencing, but not in cells where SLC37A2 was silenced (Figure 2.7A). This suggested that G6PC3 would potentially also regulate additional TGF- β -mediated cellular events in U87 cells. Thus, TGF-β-induced cell chemotaxis was next assessed. Interestingly, whereas TGF- β effectively triggered migration in siScrambled-transfected cells (Figure 2.7B,

left panel), whereas cells were found unresponsive to TGF- β when G6PC3 or SLC37A4 were silenced (Figure 2.7B, middle and right panels).

2.5 Discussion

Glioblastoma (GBM) is a hypoxic and aggressive brain tumor associated with poor patient prognosis and limited treatment options. Such clinical manifestation is, in part, attributable to the highly adaptive mechanisms of the brain cancer cells allowing their metabolic reprogramming within a low oxygen tension tumor microenvironment. In this study, we assessed the transcript levels and the specific roles of the G6P sensing components G6PC3, SLC37A2, and SLC37A4, all found increased in clinical GBM tissues (Figure 2.1) and correlated with decreased overall patient survival (Figure 2.2). Interestingly, G6Pase components G6PC3 and SLC37A4 were also found to be involved in the GBM response to the EMT inducer TGF-β and in the acquisition of a CSC phenotype. With regards to disease progression, our findings further suggest an association between the expression levels of SLC37A4 and the transition from low-grade glioma state to GBM, adding to their angiogenic and chemoresistance phenotype.

While the importance of glucose metabolism alterations in cancer development and progression is well recognized (Lei et al. 2023), the specific implication of the G6Pase system components in GBM, particularly with regards to the intracellular conversion of G6P back to glucose, thereby regulating glucose homeostasis and providing a source of energy for cancer cells, has only been inferred within the last decade (Abbadi et al. 2014). Dysregulation in the global G6Pase system has been implicated in various cancers, including ovarian cancer and HCC (Li et al. 2014, Liu et al. 2021), but the specific contributions of the G6PC1-3 and SLC37A1-4 isoforms remains unclear. In addition, G6PC was recently reported as a poor prognosis in cervical cancer and to promote cervical carcinogenesis through EMT progression in vitro and in vivo (Zhu et al. 2022). These findings shed light over the possible clinical significance of these two G6Pase system components in GBM prognosis.

Consistent with our *in-silico* data analysis, functional experiments were performed using several established GBM-derived cell lines in which high expression of G6PC3, SLC37A2, and SLC37A4 in comparison to HepG2 hepatoma cells was observed. Specific knockdown of G6PC3 and SLC37A4 genes further altered the acquisition of a CSC phenotype, and G6PC3 as well as SLC37A4 silencing prevented the TGF-β signal transduction response that led to increased chemotaxis. TGF-β plays an important role in cell metabolism and immunity and can induce a shift in cell metabolism from oxidative phosphorylation to

aerobic glycolysis, providing a favorable environment for tumor growth (Nana, Yang & Lin 2015). TGF-β also links glycolysis and immunosuppression in GBM as studies have shown that high levels of TGF-β and of its receptors are associated with glioma malignancy and a poor prognosis (Roy, Poirier & Fortin 2018, Gong et al. 2021). TGF-β and stem cell markers were found highly expressed around necrotic areas in GBM (Iwadate et al. 2016). How G6PC3 and SLC37A4 gene silencing alters the crosstalk between G6P sensing and TGF- β signaling remains to be investigated.

Notably, the expression levels of G6PC3 and SLC37A4 were found induced in neurospheres, a 3D cell culture model that recapitulates a stem cell-like phenotype associated with increased tumorigenicity and therapeutic resistance (Gupta, Chaffer & Weinberg 2009, Venere et al. 2011, Gresseau et al. 2022). This observation suggests that transcriptional manipulation of G6PC3 and SLC37A4 levels may contribute to the chemoresistance and invasive molecular signature of GBM and of GBM-derived CSC. The knockdown of G6PC3 and SLC37A4 in neurospheres resulted in a significant reduction in the expression levels of CSC markers, including EMT biomarkers, all collectively associated with stemness, self-renewal capacity, and invasive properties of glioma stem cells (Huang et al. 2020). The impaired stemness properties and reduced invasive features upon knockdown of G6PC3 and SLC37A4 again confirm their implication in metabolic reprogramming and suggest their potential as future therapeutic targets to mitigate the aggressive behavior of GBM. Although glycolysis inhibitors are widely used to target such reprogramming, their efficacy in GBM remains unclear, especially within a hypoxic tumor microenvironment (McKelvey et al. 2021, Vallejo et al. 2019, Shi et al. 2022). Inhibition of glycolysis has also been inferred as an effective strategy to eradicate residual brain cancer stem cells that are otherwise resistant to chemotherapeutic agents in their brain-hypoxic niches (Zhou et al. 2011).

G6PC3 roles in metabolic alterations remain poorly documented. Consistent with our current study, G6PC3 expression was recently reported to be up-regulated in GBM and is a prognostic risk factor (Liu et al. 2021, Chen et al. 2023). G6PC3 deficiency in human patients has a broad clinical phenotypic spectrum that involves many organs, including the brain. Its deficiency causes neutropenia in humans and in mice and is linked to enhanced apoptosis and ER stress (Gautam et al. 2013). The expression of G6PC3 in brain astrocytes, given its low G6P hydrolyzing activity, implicates a novel function for the effective uptake of glucose by astrocytes, and was speculated to allow the ER to function as an intracellular “highway” delivering glucose from perivascular end feet to the perisynaptic processes (Müller, Fouyssac & Taylor 2018). Evidence for G6PC3 as a metabolite repair enzyme was also suggested to serve a neuroprotective

role in brain to maintain energy-dependent functions, including cognitive processes (Dienel 2020). Previously debated and discounted functions for brain G6PC3 include causing an ATP-consuming futile cycle and a nutritional role involving astrocyte-neuron glucose-lactate trafficking. Interestingly, failure to eliminate a phosphorylated glucose analog led to neutropenia in patients with SLC37A4 and G6PC3 deficiency (Veiga-da-Cunha et al. 2023). It was demonstrated that SLC37A4 and G6PC3 collaborated to destroy 1,5-anhydroglucitol-6-phosphate (1,5AG6P), a close structural analog of G6P and an inhibitor of low-KM hexokinases, which catalyze the first step in glycolysis in most tissues. Failure to eliminate 1,5AG6P appears to be the mechanism of neutrophil dysfunction and death in G6PC3-deficient mice (Hiwarkar et al. 2022).

In conclusion, our study highlights an original and underestimated potential of G6PC3 and SLC37A4 as prognostic markers and therapeutic targets in brain cancer. The upregulation of these genes in GBM tissues, and additionally in brain CSC models, further suggests their association with stemness properties and invasive characteristics. Whether G6PC3 and SLC37A4 may contribute, in part through G6P sensing processes, to the pro-angiogenic phenotype of GBM remains speculative, but would align with previous studies indicating the involvement of glucose metabolism alterations in angiogenesis regulation (Yuen et al. 2016, Niu et al. 2023). Targeting components of the G6Pase system, either involved in the G6P recognition/transport or hydrolysis within the ER, may potentially provide a novel approach to modulate angiogenesis in GBM.

Further studies are warranted to elucidate the precise molecular mechanisms underlying the functional implications of the G6Pase components G6PC3 and SLC37A4 at the protein level in GBM. The targeted delivery and immunoregulatory effects of chlorogenic acid, a potent SLC37A4 inhibitor (Belkaid et al. 2006, Patil & Gadad 2023), were recently investigated for the treatment of GBM with promising potential (Ye et al. 2020, Xue et al. 2017). Thus, exploring the therapeutic potential of targeting the G6Pase system components in preclinical models may eventually be valuable in developing novel treatment strategies for GBM. Overall, our findings shed light on a new complex interplay between glucose metabolism, brain cancer progression, and CSC biology.

2.6 Figures and legends

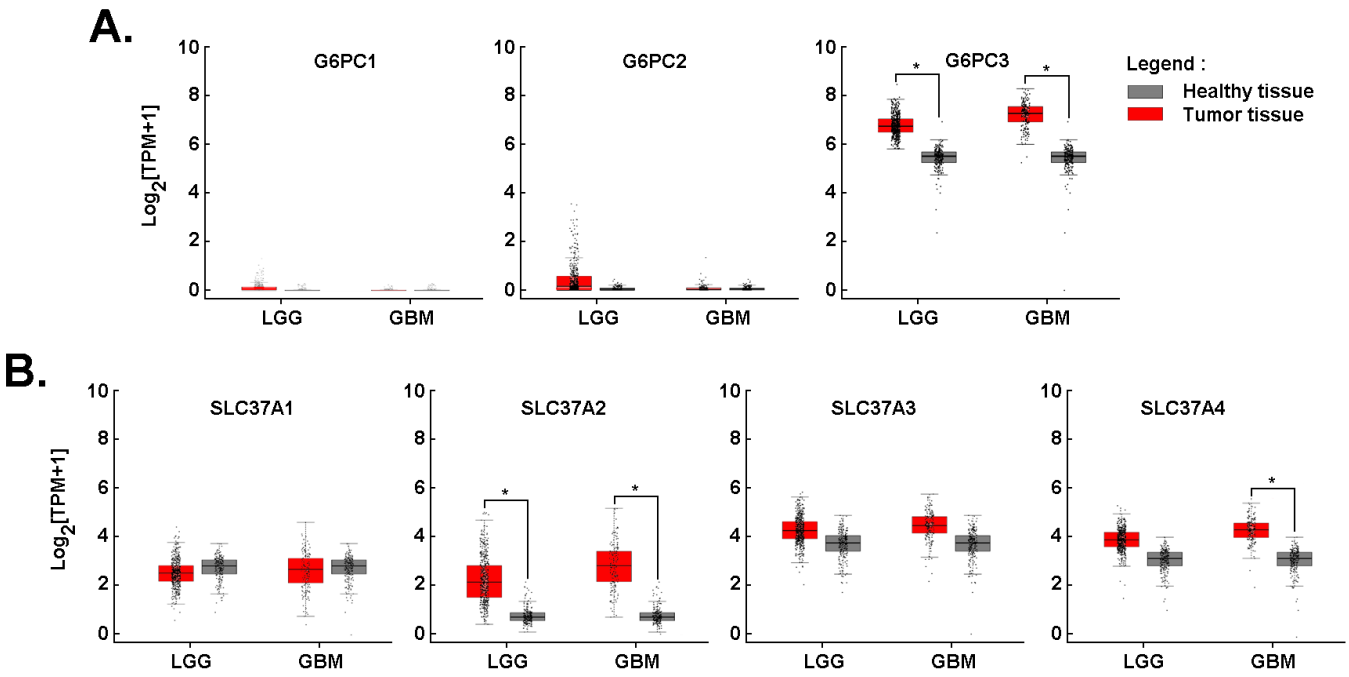

Figure 2.1: Increased gene expression of G6PC3, SLC37A2, and SLC37A4 in clinically annotated glioblastoma tumor tissues. In silico analysis of transcript levels was performed for A) three members of the G6PC family (G6PC1, G6PC2, G6PC3) and B) four members of the SLC37A family (SLC37A1, SLC37A2, SLC37A3, SLC37A4) using RNA extracted from clinical samples from glioblastoma (GBM) and low-grade glioma (LGG) (red boxes) and compared to healthy tissue (grey boxes). Probability values of 0.05 were judged significant and indicated as (*). The y-axis is labeled as "Log2 (TPM+1)". Log2 TPM is the logarithm of the Transcript Count Per Million (TPM). TPM is a normalization technique used to scale the read count per gene/transcript towards the total read count of the sequencing run in order to compensate for different sequencing depths. Log2 TPM is a commonly used metric in RNA-seq data analysis to describe the expression level of a gene in a sample.

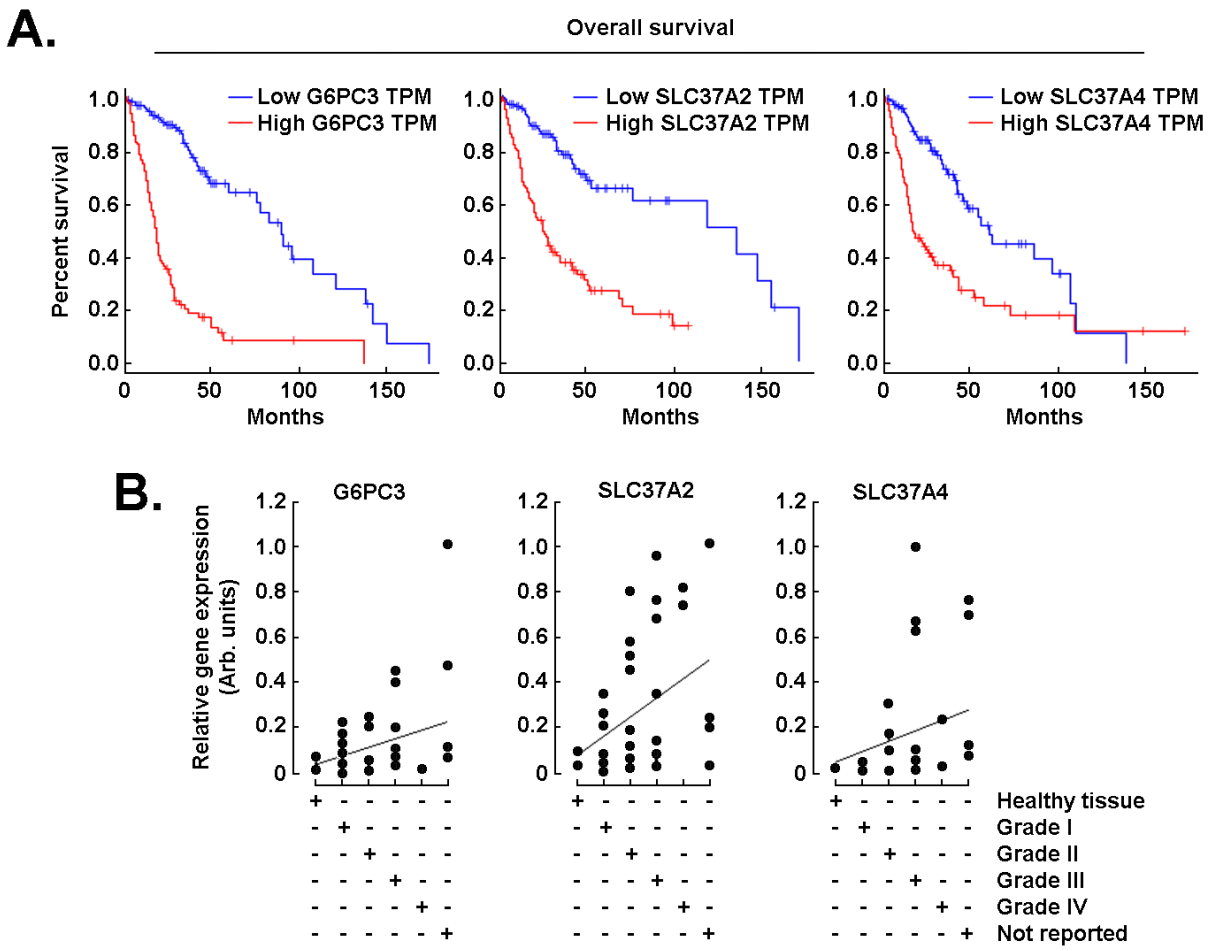

Figure 2.2: High G6PC3, SLC37A2, and SLC37A4 gene expression correlates with poor prognosis in GBM patients. A) Kaplan-Meier analysis was performed using transcriptional programs addressing a database of gene expression profiles from healthy and malignant brain cancer as described in the Methods section. The three panels show a survival plot based on a high-quality data set displaying a full analysis of G6PC3, SLC37A2, and SCL37A4. Blue lines show patients with gene expression below median levels (low expression), whereas red lines show patients with gene expression above median (high expression). B) TissueScan™ brain cancer and normal tissue cDNA arrays from 43 clinical samples covering four stages of brain cancer were used to assess G6PC3, SLC37A2, and SCL37A4 gene expression levels.

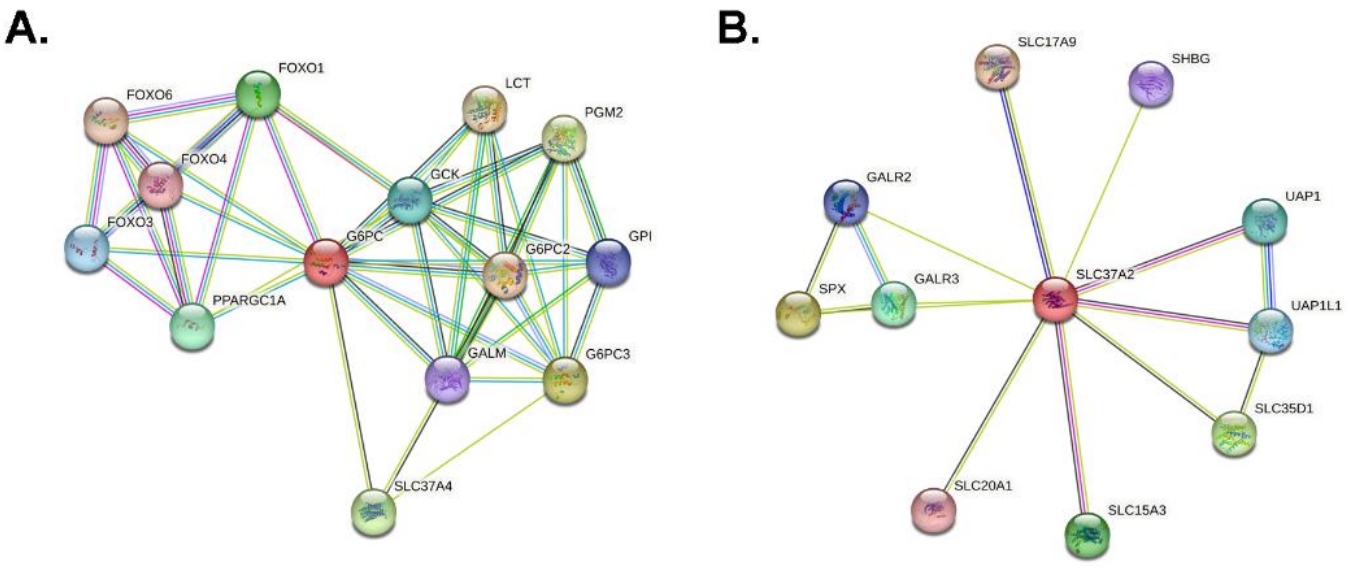

Figure 2.3: Protein-protein interaction (PPI) network predicts G6PC3 interrelationship with SLC37A4, but not SLC37A2. Indirect target proteins of the core A) G6PC3- and B) SLC37A2-associated network were built by STRING database as described in the Methods section.

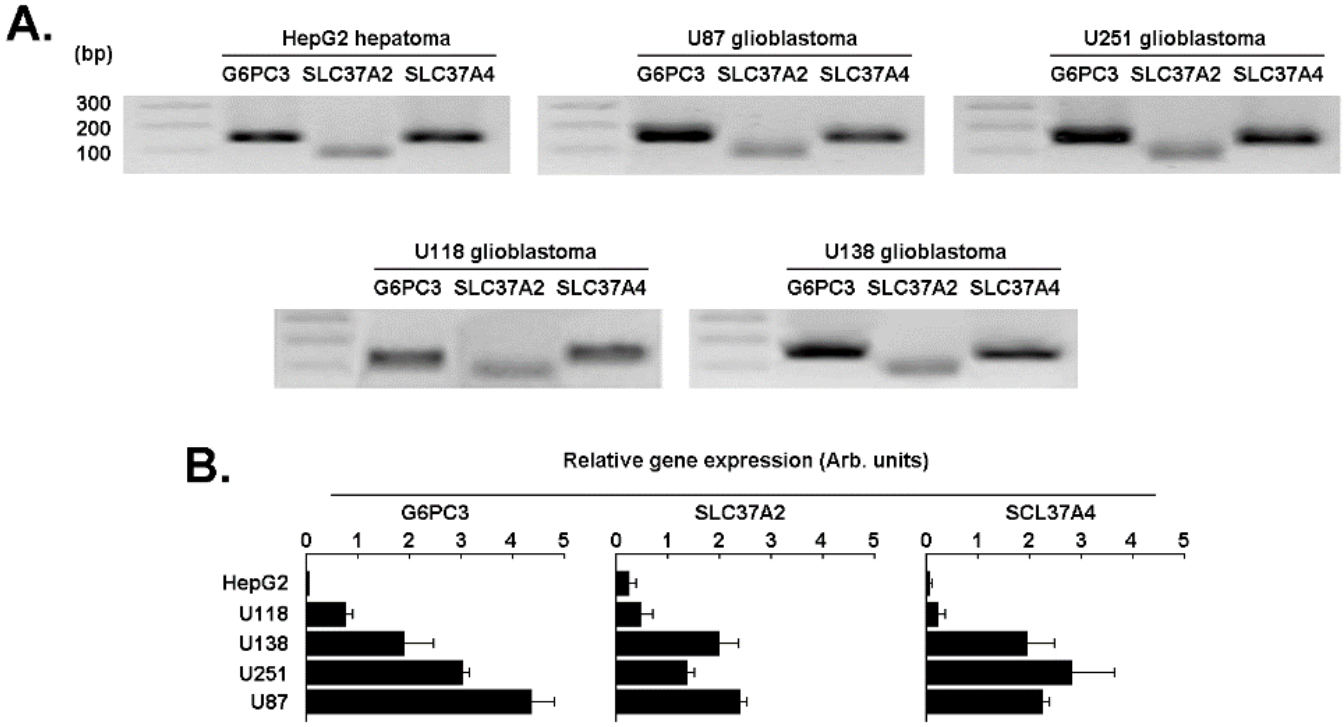

Figure 2.4: Expression of the G6PC3, SLC37A2, and SLC37A4 are increased in several human glioblastoma cell lines. Total RNA was extracted from four different human glioblastoma cell lines (U87, U118, U251, and U138) and the gene expression level for G6PC3, SLC37A2, and SLC37A4 analyzed by RT-qPCR and compared to the human HepG2 hepatoma cell line. A) Primers validation and single amplicon products were confirmed using agarose gel electrophoresis for all cell lines tested. B) The gene expression levels of the three selected genes was analyzed and quantified by qPCR as described in the Methods section. Triplicates from a representative quantification, out of three independent experiments, is shown.

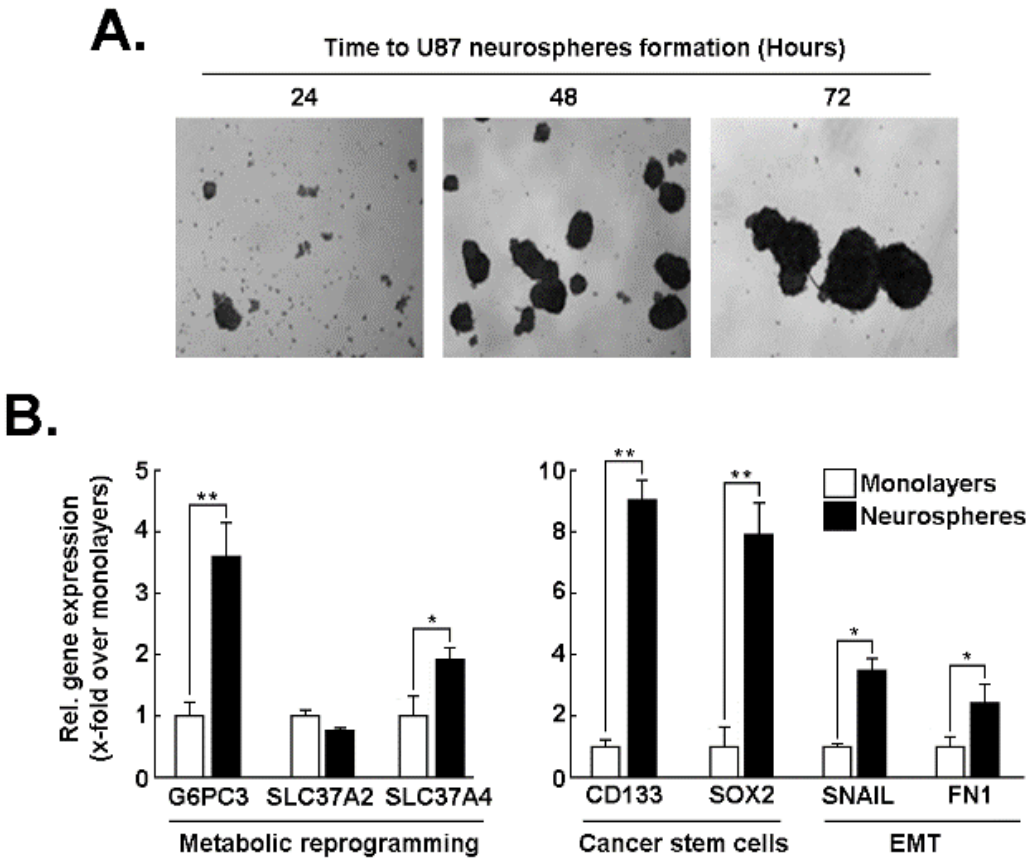

Figure 2.5: G6PC3 and SLC37A4 levels are induced in CD133/SOX2-positive U87-derived neurospheres. Human U87 glioblastoma cell monolayers were cultured with the Tumorsphere Medium Xf with SupplementMix for up to 72 hours. A) Phase contrast pictures were taken to monitor spheroids formation at the indicated time. B) Total RNA was extracted from either adherent monolayers (white bars) or from neurospheres (black bars) cultured for 72 hours. RT-qPCR analysis was next used to study the expression of G6PC3, SLC37A2, SLC37A4, CSC markers CD133 and SOX2, as well as EMT markers FN1 and Snail. Triplicates from a representative quantification, out of three independent experiments, is shown.

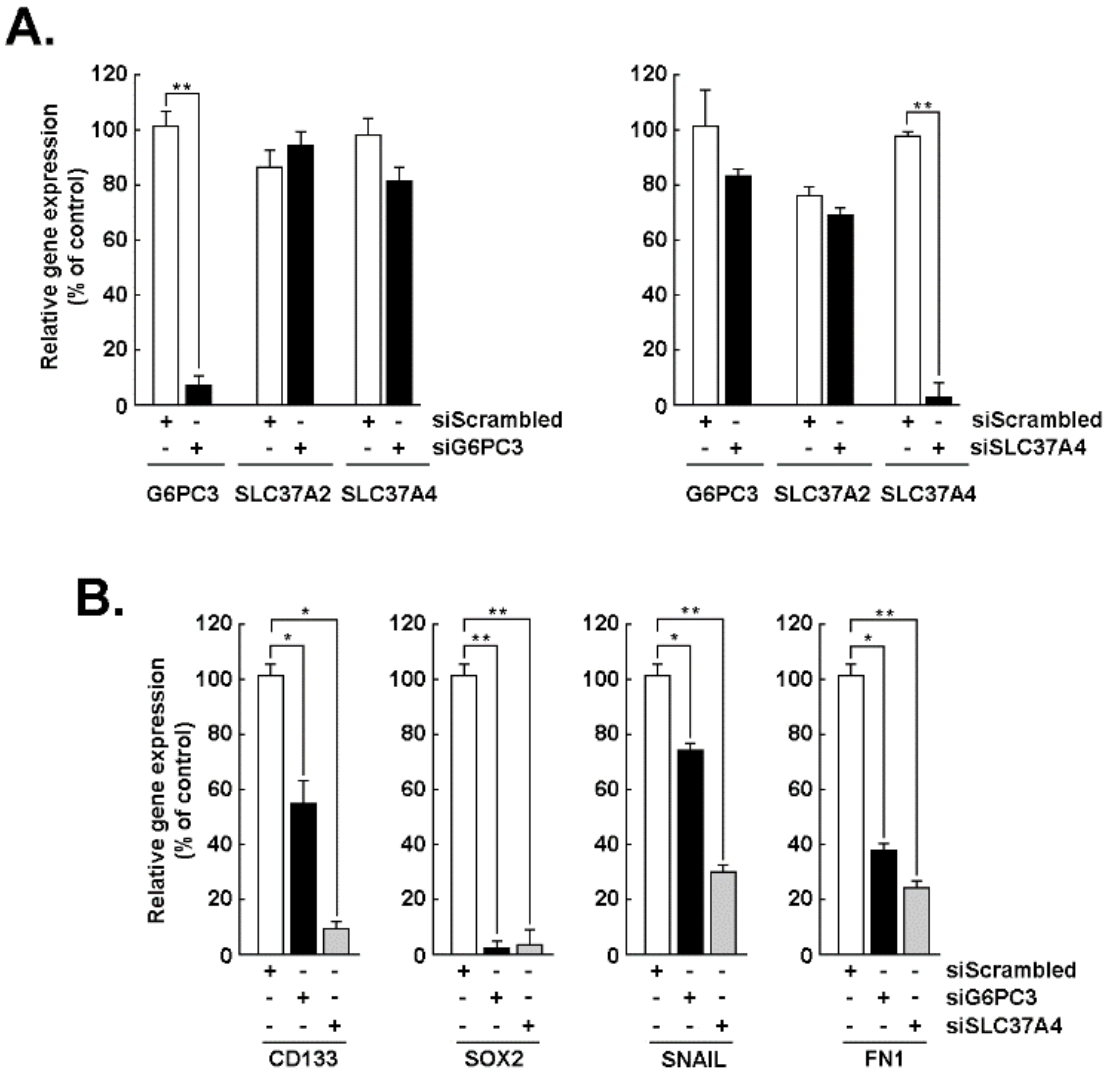

Figure 2.6: Silencing of G6PC3, SLC37A2, and SLC37A4 alters the acquisition of a CSC phenotype and expression of EMT markers. A) U87 glioblastoma monolayers were transiently transfected with a scrambled sequence (siScrambled, white bars) or siRNA directed against G6PC3 (siG6PC3, left panel, black bars), or SLC37A4 (siSLC37A4, right panel, black bars). Total RNA was then extracted, and gene silencing efficiency validated using RT-qPCR. B) After 24 hours of transfection, U87 cells were cultured with the Tumorsphere Medium Xf with SupplementMix for 72 hours. Total RNA was extracted from neurospheres and selected CSC and EMT markers analyzed by RT-qPCR in siScrambled (white bars), siG6PC3 (black bars), and siSLC37A4 (grey bars).

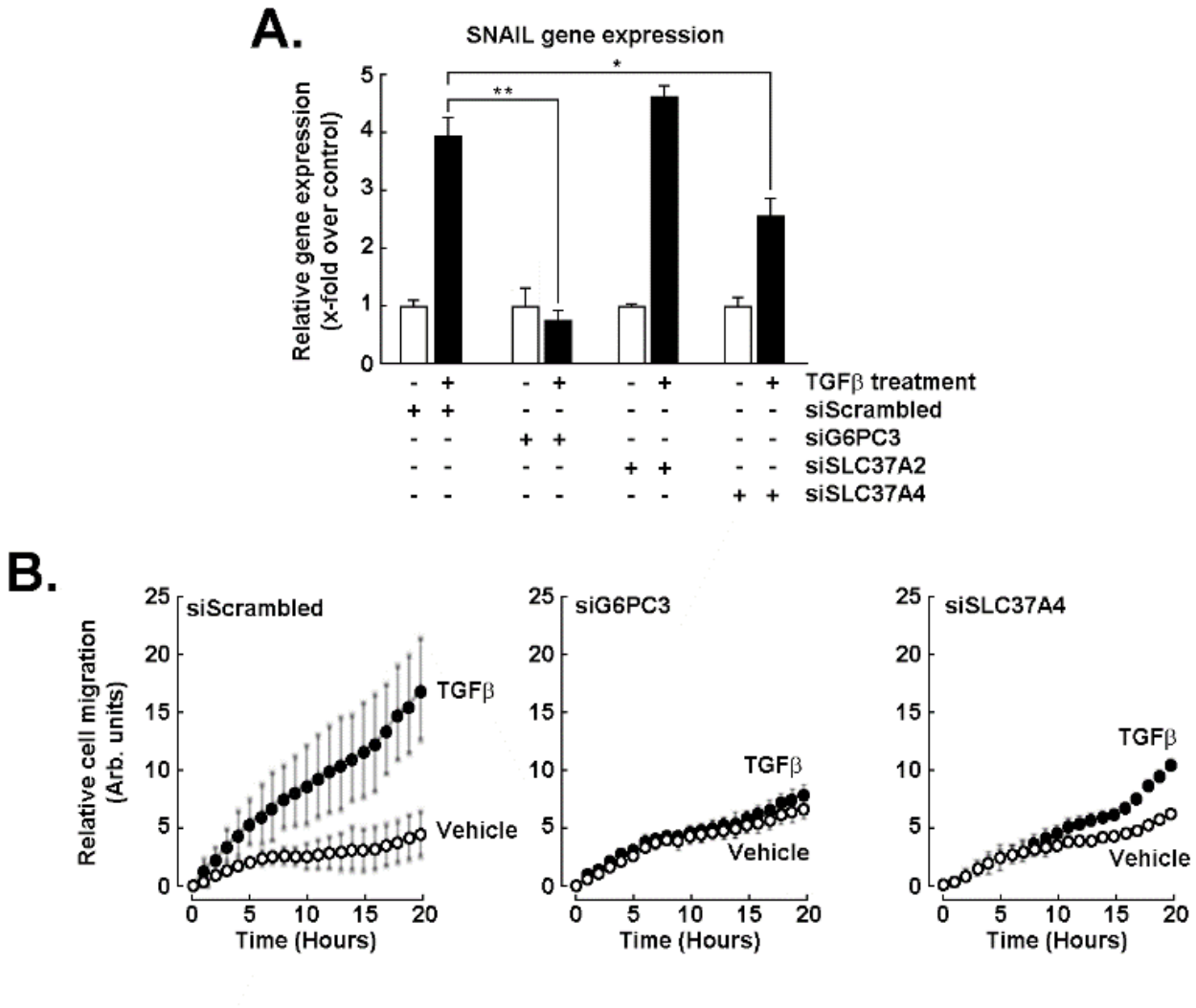

Figure 2.7: Evidence for G6PC3 and SLC37A4 involvement in the chemotactic response of U87 glioblastoma cells to TGF-β. Transient siRNA-mediated G6PC3, SLC37A2, and SLC37A4 gene silencing was performed in U87 glioblastoma cells. A) Cells' response to 10 nM TGF-β treatment for 24 hours was next monitored by RT-qPCR through the assessment of SNAIL gene expression. B) Cell chemotaxis was assessed in unstimulated (vehicle, open circles) or in response to 10 nM TGF-β (closed circles) as described in the Methods section.

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CHAPITRE 3

Discussion

The complex and intricate mechanism of cell metabolic reprogramming holds significant promise in our understanding of diseases, in particular cancer. Metabolic reprogramming facilitates various hallmark capabilities of cancer cells, notably their rapid proliferation, survival, and invasiveness (Hanahan & Weinberg, 2011). More importantly, it plays a pivotal role in adapting oncogenic cells to challenging conditions such as hypoxia, thereby perpetuating tumor growth and treatment resistance (Mohyeldin et al., 2010). A specific enzymatic system implicated in these metabolic shifts, and therefore of great interest to researchers, is the glucose-6-phosphatase (G6Pase) system. Our research aimed to examine the role of the G6Pase system in 1) GBM tissues, 2) GBM response to the EMT inducer TGF-β, and 3) the acquisition of a CSC phenotype.

Inherited metabolic disorders, often life threatening, arise from gene mutations in metabolic enzymes that alter their biosynthesis, assembly, or activity (Boyer et al., 2015). In normal cells, calorigenic nutrients undergo catabolic reactions in the presence of oxygen, culminating in ATP production. Dysregulation of these enzyme activities, whether by mutation or altered expression, leads to metabolic disorders and has also been linked to cancer development (Sreedhar & Zhao, 2018). A significant driver of such enzymatic dysregulation in cancer cells is the need to thwart nutrient depletion and support continued proliferation (Sreedhar & Zhao, 2018). However, the underlying causal relationships remain mired in complexity.

By exploiting the altered expression of G6PC, tumor cells can reprogram glucose metabolism to support their proliferative agenda. The accumulation of glucose-6-phosphate (G6P), especially in contexts where G6PC is under-expressed, boosts glucose metabolism through pathways like the hexose monophosphate (HMP) shunt, which drives tumor cell survival and growth (Wang et al., 2012). Conversely, overexpression of G6PC in GBM leads to increased glycolysis rates (Abbadi et al., 2014). This intricate balance, orchestrated through pathways like the FOXO1 in ovarian cancer and HIF1α and STAT3 in GBM, underscores the profound impact of metabolic reprogramming on cancer cell dynamics (Guo et al., 2015 & Abbadi et al., 2014).

Adding layers to this complexity, the G6P transporter (G6PT/SLC37A4) is a crucial partner in the G6Pase system, working together with the G6PC to ensure glucose homeostasis (Chou et al., 2010 & Khan et al.,

2015). G6PT's role is especially highlighted in GBM, where it inhibits cancer cell proliferation and regulates cell survival, likely acting as a metabolic "bioswitch" (Belkaid et al., 2007). Intriguingly, its involvement in calcium-mediated signaling underscores its multi-faceted roles in cancer cell regulation (Belkaid et al., 2006).

The significance of changes in glucose metabolism during carcinogenic processes is widely acknowledged (Lei et al., 2023). However, the precise involvement of the components of the G6Pase system in GBM, specifically in relation to the intracellular transformation of G6P back into glucose, thereby regulating the balance of glucose within cells and serving as a source of energy for cancer cells, has only been deduced in the past ten years (Abbadi et al., 2014). Recent studies reveal the perturbed expression of G6Pase in several cancers. While downregulated in gluconeogenic tissue cancers like HCC (hepatocellular carcinoma) and RCC (renal cell carcinoma), it is upregulated in non-gluconeogenic tumors such as GBM (Guo et al., 2015). This upregulation promotes tumor survival and invasion under stress, fostering resistance against treatments like 2-deoxyglucose (Guo et al., 2015). G6Pase upregulation has been linked to cancer progression through various mechanisms. It has been shown to promote tumor growth by activating the pentose phosphate pathway (PPP) and increasing glucose flux, providing precursors for biosynthesis and reducing equivalents for antioxidant defense (Rao, 2015). G6Pase-mediated increase in *de novo* NADP+ biosynthesis has also been found to promote antioxidant defense and tumor metastasis (Zhang, 2022). Furthermore, G6Pase upregulation has been associated with poor prognosis in bladder cancer, with high expression levels correlating with increased tumor stage and worse survival rates (Chen, 2018). These findings suggest that G6Pase upregulation may play a significant role in cancer progression and could be a potential target for cancer therapy. Moreover, experiments have highlighted the potential therapeutic targeting of G6PC and the SLC37A family to modify cancer cell survival and phenotype, although the precise implications of the G6PC1-3 and SLC37A1-4 isoforms have yet to be fully elucidated.

Here, molecular and cellular tests were conducted utilizing several well-characterized GBM-derived cell lines, in line with our computational data assessment. We observed a notable expression of G6PC3, SLC37A2, and SLC37A4 compared to HepG2 hepatoma cells. HepG2 cells have been extensively used in studies related to G6Pase and cancer. G6PD deficiency in HepG2 cells increased susceptibility to H(2)O(2)-induced apoptosis, suggesting a potential role of G6PD in cancer cell survival(Lin, 2010). The role of hexokinase II, which is overexpressed in cancer cells, in promoting cancer cell survival and proliferation (Mathupala, 2006). The investigation in regulation of glucose 6-phosphatase in HepG2 cells shows distinct

hormone stimulation and counteraction by insulin on the expression of the two components of glucose 6-phosphatase and demonstrates that protein kinase A phosphorylates hepatocyte nuclear factor-6 and stimulates glucose-6-phosphatase catalytic subunit gene transcription (Li, 2000; Streeper, 2001). These studies collectively suggest that HepG2 cells can serve as a useful control in the study of glucose-6-phosphatase and cancer, providing a stable and well-characterized cell line for comparison. Explicit suppression of G6PC3 and SLC37A4 genes altered the adoption of a CSC characteristic, while repressing G6PC3 and SLC37A4 expression obstructed the TGF-β signal transmission response. TGF-β, crucial for cell metabolism and immunity, can cause a metabolic shift from oxidative phosphorylation to aerobic glycolysis, fostering a conducive environment for tumor progression (Nana et al., 2015). Moreover, TGF-β associates glycolysis with immunosuppression in GBM, as research indicates that elevated TGF-β levels and its receptors correlate with glioma malignancy and unfavorable outcomes (Gong et al., 2021). Accordingly, TGF-β and stem cell marker expression were detected around necrotic zones in GBM (Iwadate et al., 2016). The mechanism by which the silencing of G6PC3 and SLC37A4 genes affects the interaction between G6P sensing and TGF-β signaling warrants further exploration.

Epithelial-mesenchymal transition (EMT) is a thoroughly studied phenomenon that augments the motility and invasiveness of malignant cells. This process plays a crucial role in the progression of tumor metastasis (Maren & Gerhard, 2016). Previous investigations have demonstrated that G6PC can facilitate the metastasis of GBM (Abbadi et al., 2014). Additionally, it has been found that G6PC hinders the migration of cervical cancer cells by regulating EMT. The findings presented in this study provide valuable insights into the potential clinical relevance of the two components of the G6Pase system in the prognosis of GBM. The induction of G6PC3 and SLC37A4 expression levels was discovered in neurospheres, a 3D cell culture model that replicates a stem cell-like phenotype that is connected to an increased ability to form tumors and resistance to therapy (Venere et al., 2011; Gresseau et al., 2022). This finding suggests that the manipulation of transcriptional activity in G6PC3 and SLC37A4 levels might contribute to the molecular characteristics of GBM and GBM-derived CSC, such as chemoresistance and invasiveness. When G6PC3 and SLC37A4 were knocked down in neurospheres, there was a significant decrease in the expression levels of CSC markers, including EMT biomarkers, which are all collectively associated with the characteristics of stemness, the capacity for self-renewal, and the invasive properties of glioma stem cells (Huang et al., 2020).

Hypoxia, which signifies diminished oxygen levels, is a widely recognized and all-encompassing characteristic of the tumor microenvironment (Brown & Wilson, 2004). It has been correlated with heightened tumor aggressiveness and a decreased susceptibility to traditional oncological therapies. Prior investigations have yielded valuable knowledge regarding alterations in the transcriptome and proteome induced by hypoxia. The diminished stemness attributes and lessened invasive qualities following the knockdown of G6PC3 and SLC37A4 reaffirm their involvement in metabolic reprogramming and suggest their potential as upcoming therapeutic targets to temper the hostile nature of GBM (Kucharzewska et al., 2015). While inhibitors of glycolysis are broadly utilized to target this reprogramming, their effectiveness in GBM continues to be ambiguous, particularly within a hypoxic tumor microenvironment (McKelvey et al., 2021 & Shi et al., 2022). Additionally, obstructing glycolysis has been suggested as a potent strategy to eliminate remaining brain CSCs, which are otherwise impervious to chemotherapeutic agents in their brain-hypoxic niches (Zhou et al., 2011).

The role of G6PC3 in modifying metabolic pathways has not been extensively documented to date. In alignment with our ongoing study, the expression of G6PC3 has been recently noted to elevate in GBM, identifying it as a prognostic risk factor (Liu et al., 2021). Intriguingly, the lack of G6PC3 in human subjects presents a wide-ranging clinical phenotype, impacting various organs, including notably the brain. A deficiency in G6PC3 triggers neutropenia, a condition characterized by an abnormally low count of neutrophils, both in humans and mice. This condition is associated with an increase in apoptosis and endoplasmic reticulum (ER) stress (Gautam et al., 2013).

When considering the expression of G6PC3 in brain astrocytes, its minimal G6P hydrolyzing activity insinuates a previously unexplored function: facilitating the efficient absorption of glucose by astrocytes. This notion was further hypothesized to enable the ER to serve as an intracellular "highway," transporting glucose from perivascular endfeet to the perisynaptic processes (Müller et al., 2018). Additional evidence points towards G6PC3 acting as a metabolite repair enzyme, potentially offering a neuroprotective function within the brain to uphold energy-dependent functions, inclusive of cognitive processes (Dienel, 2020). In earlier discussions, some debated and eventually disregarded roles for brain G6PC3 included inducing an ATP-consuming futile cycle and a nutritional role involving astrocyte-neuron glucose-lactate trafficking.

A particularly notable observation was that the inability to eradicate a phosphorylated glucose analog led to neutropenia in patients who were deficient in SLC37A4 and G6PC3 (Veiga-da-Cunha et al., 2023). Detailed studies demonstrated that SLC37A4 and G6PC3 worked in conjunction to degrade 1,5-anhydroglucitol-6-phosphate (1,5AG6P), a structural analog of G6P that closely resembles it and acts as an inhibitor of low-KM hexokinases. These hexokinases facilitate the initial step in glycolysis across numerous tissues. The incapacity to eliminate 1,5AG6P is projected to be the underlying mechanism behind neutrophil dysfunction and death in G6PC3-deficient mice (Hiwarkar et al., 2022). This rich vein of inquiry continues to unravel the multifaceted roles and implications of G6PC3 and SLC37A4 in metabolic reprogramming, presenting numerous avenues for further exploration and potential therapeutic intervention in the context of GBM and other related conditions.

During an experiment, it was observed that the behavior of SLC37A2 in both gene and protein levels is different from G6PC3 and G6PT. Contrary to SLC37A4 (G6PT), other solute carrier family members are less known for their biological role. Research has unveiled crucial insights into SLC37A2, revealing its attributes and functionality related to G6P transport. It was demonstrated that SLC37A2 functions as a phosphate (Pi)-linked G6P antiporter, capable of conducting exchanges of G6P:Pi and Pi:Pi, akin to its counterpart G6PT (Pan et al., 2011). Despite these similarities, notable distinctions emerge when the transport activity of SLC37A2 is compared to G6PT. For one, the transport activities of SLC37A2 are not susceptible to inhibition by chlorogenic acid (Pan et al., 2011). Secondly, SLC37A2 does not couple functionally with either G6PC1 or G6PC3 to mediate microsomal G6P uptake, highlighting distinct operational differences from G6PT (Pan et al., 2011). Furthermore, although there is evidence of another microsomal G6P transporter activity in human cell lines that remains insensitive to chlorogenic acid inhibition (Leuzzi et al., 2001), it is presently indeterminate whether these activities are attributed to SLC37 family proteins, including SLC37A2. An intriguing aspect of SLC37A2, along with SLC37A1 and SLC37A3, is its sharing of a 17-residue signature motif (ProSite PDOC00726) with G6PT and other related proteins (Chou & Mansfield, 2014). While SLC37A2 and SLC37A1 diverge from the consensus sequence in five positions, they uphold residues with akin characteristics. Additionally, while it is evident that multiple sugar-phosphate transporters operate within the ER, the physiological substrate for SLC37A2, in particular, still beckons further exploration and understanding. Consequently, while the detailed functional implications and potentials of SLC37A2 have begun to be mapped, further investigative work is paramount to decipher the exact mechanisms, substrate specificities, and therapeutic potentials encapsulated within its activity (Chou & Mansfield, 2014).

Conclusion

The research presented had three main objectives: The first was to appraise G6PC and SLC37A Family genes contributing to GBM. Since, the *in-silico* study and following validation experiments showed upregulation in GBM tissues and GBM cell lines. The upregulation of G6PC3 and SLC37A4 among these genes in GBM tissues and cell lines validated by *in-silico* study led us to expand our experiments with the second objective. Therefore, we used neurospheres to recapitulate the brain CSC phenotype and studied the expression level of these genes to get deeper in their contribution to the acquisition of a CSC phenotype. Finally, to identify the role of these genes in GBM survival, invasion and migration transient transfection method using siRNA sequences was conducted alongside migration assay, three-dimensional neurosphere formation, and other Techniques in the presence or absence of TGF-β.

In conclusion, our investigation underscores a distinctive and previously underappreciated potency of G6PC3 and SLC37A4 as pivotal prognostic markers and therapeutic conduits in the realm of brain cancer. The discerned upregulation of these specified genes, not only in GBM tissues but also intriguingly in brain CSC models, propels further speculation regarding their association with properties of stemness and the invasive hallmarks of the malignant cells. The conjecture that G6PC3 and SLC37A4 might contribute—potentially via mechanisms related to G6P sensing—to the pro-angiogenic phenotype inherent to GBM still veils itself in speculation. However, this theory does dovetail with prior research that suggests a consequential relationship between alterations in glucose metabolism and the regulation of angiogenesis (Yuen et al., 2016; Niu et al., 2023).

Delving into the components of the G6Pase system, especially those entangled in the intricacies of G6P recognition, transport, or hydrolysis within the ER, could pave the way towards the identification of innovative strategies to modulate angiogenesis in GBM.

Moreover, a further, more nuanced exploration is required to elucidate the exact molecular underpinnings that underscore the functional impacts of G6Pase components G6PC3 and SLC37A4, particularly at the protein level, in GBM. The exploration into targeted delivery and the immunoregulatory impacts of chlorogenic acid—a notably potent inhibitor of SLC37A4 (Patil & Gadad, 2023)—has recently been delved into for GBM treatment, unveiling a realm of promising potential (Ye et al., 2020; Xue et al., 2017). As such, a rigorous exploration into the therapeutic potential of strategically targeting G6Pase system components

through preclinical models could open up a new frontier in designing innovative treatment strategies for GBM.

In a holistic view, our findings peel back a layer, revealing a complex and intricate interplay amongst glucose metabolism, the progressive trajectory of brain cancer, and the multifaceted biology of CSCs. This interplay suggests a pathway through which we might intercept and manipulate the disease trajectory, offering a glimmer of hope and a potential new avenue toward effective therapeutic interventions in the treatment of formidable brain malignancies like GBM. In pursuit of evolving these findings from the bench to bedside, the continuum of research must embrace multidisciplinary and collaborative efforts to navigate the complexities of tumor biology and therapeutic response.

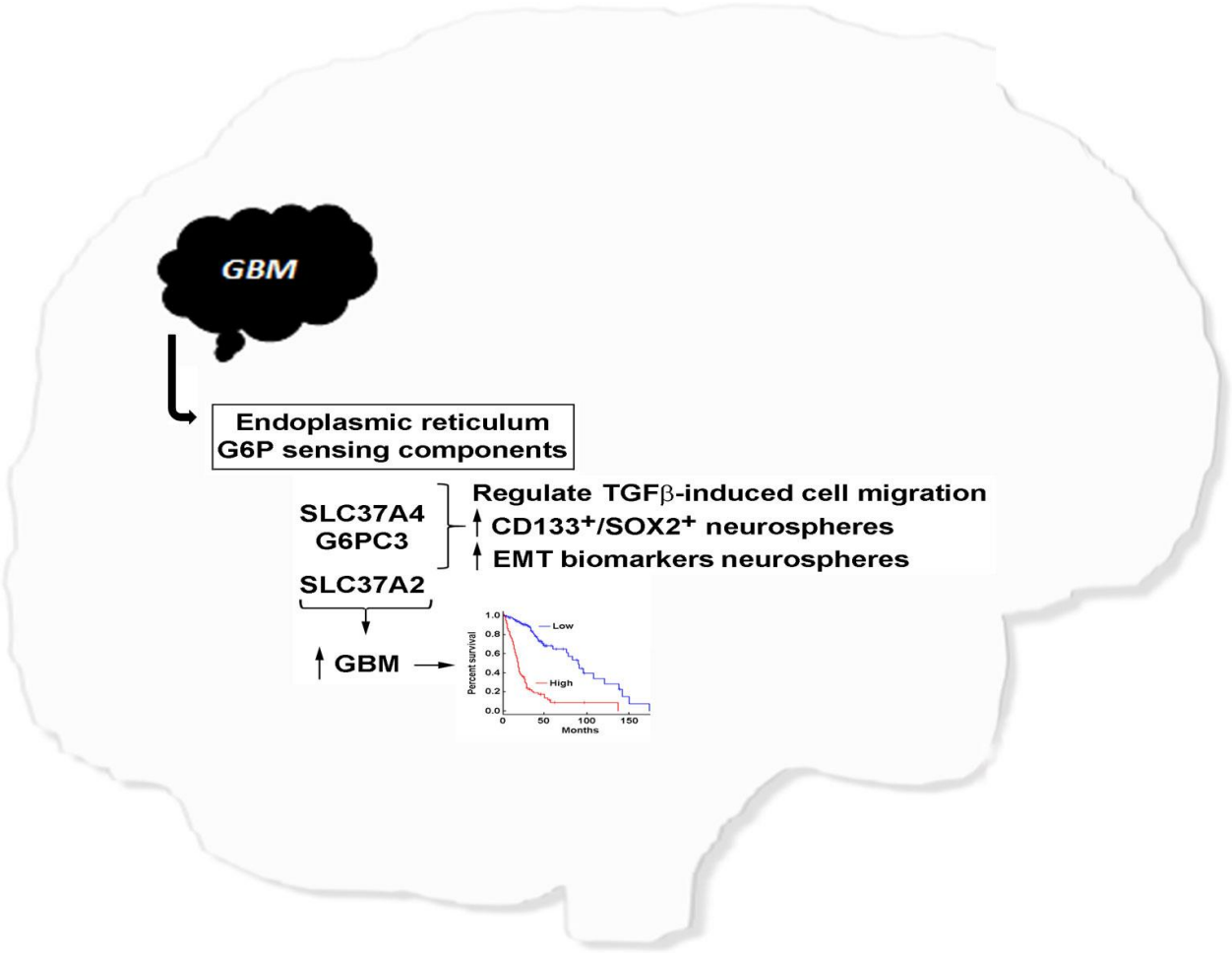

Figure 3.1: Graphical abstract.

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