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THE ROLE OF GLUTAMINE AND MEVALONATE PATHWAY IN AFFECTING THE STEMNESS AND VIABILITY OF GLIOBLASTOMA STEM CELLS

Naima Hammoudi

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**THE ROLE OF GLUTAMINE AND MEVALONATE PATHWAY IN AFFECTING THE
STEMNESS AND VIABILITY OF GLIOBLASTOMA STEM CELLS**

By

Naima Hammoudi, B.S.

APPROVED

Dr. Peng Huang, MD., Ph.D. Supervisory Professor

Varsha Gandhi, Ph.D.

Shiaw-Yih Lin, Ph.D.

Honami Naora, Ph.D.

Zahid Siddik, Ph.D.

APPROVED:

Dean, The University of Texas

Graduate School of Biomedical Sciences at Houston

**THE ROLE OF GLUTAMINE AND MEVALONATE PATHWAY IN AFFECTING THE
STEMNESS AND VIABILITY OF GLIOBLASTOMA STEM CELLS**

**A
DISSERTATION**

**Presented to the Faculty of
The University of Texas
Health Science Center at Houston**

and

**The University of Texas
MD Anderson Cancer Center
Graduate School of Biomedical Sciences**

In Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

By

Naima Hammoudi, B.S.

Houston, Texas

August, 2014

Dedication

To my parents.

Acknowledgements

I would like to express my gratitude to my mentor, Dr. Huang for his guidance through this journey.

I am thankful to all my committee members: Dr. Siddik, Naora, Gandhi, Lin, Calin, Claret for offering me their mentorships, guidance and supports to complete my project. Your criticisms were always well founded, and I learnt a great deal from them. As Khalil Gibran wrote, “The teacher who is indeed wise does not bid you to enter the house of his wisdom but rather leads you to the threshold of your mind.”

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I am humbled and grateful to have been given the efficient means to accomplish my dreams, goals, and desires in biomedical research, and I am determined to continue to expand our scientific knowledge for the long-term goal of developing new medicines to placate our fellow mankind.

THE ROLE OF GLUTAMINE AND MEVALONATE PATHWAY IN AFFECTING THE STEMNESS AND VIABILITY OF GLIOBLASTOMA STEM CELLS

Naima Hammoudi, B.S.

Supervisory Professor: Peng Huang, M.D., Ph.D.

Glioblastoma (GBM) remains the deadliest form of brain tumors. The poor prognosis of glioblastoma patients is associated with a high rate of relapse after therapy. It has been suggested that the presence of cancer stem cells, which are relatively resistant to radiation and chemotherapy, may play a significant role in the recurrence of brain tumor. Understanding the biological property of glioblastoma stem cells is important to develop effective therapeutic strategies for glioblastoma. *In vitro*, glioblastoma stem cells cultured in serum-free medium form self-renewing neurospheres, express the neural stem marker CD133, and are highly tumorigenic. On the other hand, in the presence of fetal bovine serum (FBS), the glioblastoma stem cells undergo differentiation.

In this study we used glioblastoma stem cells, GSC11 and GSC23, previously isolated from glioblastoma patients and expressed stem cell markers CD133, Olig2 and SOX2, to explore their bioenergetics by monitoring the oxygen consumption as an indication of mitochondrial respiration cultured in serum-free medium in comparison with that incubated in medium containing FBS. We found that GSC11 and GSC23 stem cells exhibited low mitochondrial respiration when cultured in stem cell medium. Upon exposure to FBS mitochondrial respiration increased significantly. Metabolic changes were also observed. Furthermore, we found that glutamine uptake was higher in GBM stem cells compared with FBS induced cells. Additionally, treatment of the cells with Compound 968, a glutaminase inhibitor, depleted the cells from the CD133 marker, slowed proliferation and limited growth on soft agar. Moreover, GBM stem cells showed an increase in the expression of genes related to

the mevalonate pathway. We also found that simvastatin, an inhibitor of the mevalonate pathway, induced GBM stem cells death.

This study showed mitochondria metabolic reprogramming of GBM stem cells during differentiation. It also showed the importance of glutamine in maintaining CD133 expression and GBM stem cells growth. Lastly, the study showed that mevalonate pathway is a target to eliminate GBM stem cells.

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Abbreviations

AMPK	AMP-activated protein kinase
ATP	adenosine triphosphate
bFGF	fibroblast growth factor
c-MYC	V-myc avian myelocytomatosis viral oncogene homolog
CSC	cancer stem cells
ECT	electron transport chain
EGF	endothelial growth factor
FPP	arnasyl pyro phosphate
GBM	glioblastoma stem cells
GFAP	glial fibrillary acid protein
GGPP	geranygreanyl pyro phosphate
GLS1	glutaminase
HIF	hypoxia inducible factor
HK	hexokinase
LDH-A	lactate dehydrogenase A
LIF	leukemia inhibitory factor
MtDNA	mitochondria DNA
mTOR	mammalian target of rampamycin
NADPH	nicotinedine adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
NRF1	Nuclear respiratory factor-1
Oxhpos	oxidative phosphorylation
PDK1	pyruvate dehydrogenase kinase 1
PGC1- α	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
PTEN	Phosphatase and tensin homolog
ROS	reactive oxygen species
SCID	severe combined immune-deficient
SOX2	sex determining region Y-Box2
SREBP	sterol regulatory element-binding proteins
TCA	citric acid cycle
TFAM	mitochondrial transcription factor A
TGF- β	transforming growth factor- β
TUJ1	neuron-specific class III beta-tubulin

CHAPTER 1. Introduction

1.1 Cancer stem cells hypothesis

Neoplasms are typically heterogeneous being comprised of different cell populations. Different cells within the primary tumor exhibit differential capacities in proliferation and differentiation as well as formation of the original tumor or its regeneration [1]. Thus, two models have been investigated to explain the tumor heterogeneity: the clonal evolution and the cancer stem cells models. Both models provide potential cellular mechanistic to the functional heterogeneity of tumors. In the clonal evolution model individual clones of tumor cells acquire a selective advantage after accumulating over time genetic and epigenetic alterations and out-compete other clones. Clonal evolution may generate genetic heterogeneity that would result in phenotypic and functional differences between cancer cells [2]. Instead, the cancer stem cell model introduce a hierarchical organization including slowly proliferating cells, rapidly proliferating cells, and terminally differentiated cells with the cancer stem cells on the apex of the hierarchy [3]. Moreover, it is thought that the population of cancer stem cells within the tumor is responsible to sustain the tumor growth.

Cancer stem cells (CSC) refer to a subset of tumor cells with unlimited self-renew, and differentiation capacities, allowing them to continually generate cells with phenotypic heterogeneity similar to the parental tumor [3, 4]. Self-renewal property is conferred by a symmetrical and asymmetrical division, consequently driving a sustained growth of the tumor. In addition, cancer stem cells are able to differentiate into multilineage. For example, glioblastoma stem cells are able to differentiate into neural and glial cells, but they also differentiate to functional endothelial cells [5].

The existence of cancer stem cells was first demonstrated in acute myeloid leukemia AML. A small subset of cells $CD34^+/CD38^-$ was identified to able to generate leukemia when transplanted into non-obese diabetic/severe combined immunodeficient mice (NOD/SCID),

whereas the inoculation of thousand-fold higher numbers of cells not bearing the marker did not [3, 6]. Importantly, the CD34 cell surface immunophenotype is also associated to normal hematopoietic stem cells, suggesting that normal stem cells makers might play a role in cancer development.

Similarly, the existence of cancer stem cells was proposed in solid tumors. Hence, Al-Hajj and colleagues were able to isolate breast cancer stem cells using the marker present in the surface of normal mammary stem cells CD44. As few as 100 cells from this population were capable to reform the tumor when injected into immunodeficient mice, whereas a higher number of CD44⁺ population failed to form tumors after reinjection [7]. In brain tumors cells have been identified using an apical plasma membrane protein expressed in embryonal plasma structure, the CD133 (prominin) [8]. In a study done by Chen et al. supported the adhesion of glioblastoma to a hierarchical model comporting cancer stem cells, in thhe study patients tumor were compromised by three types of cells; type I, a mixture of highly aggressive cells, Type III give rise to slow growing lesions of CD133⁻ cells, and a third population that is not tumorigenic [9]. Subsequently, using different markers (table), cancer stem cells have been identified in variety of tumors: colon [10], head and neck [11] , pancreatic [12], melanoma [13], hepatic [14], lung [15], prostate [16] and ovarian tumors [17].

Whereas the identification of specific surface markers has enabled the isolation and characterization of CSCs, controversies have arisen about the internal phenotype differences regarding the efficacy of these markers in identifying all cancer stem populations. Hence, cancer stem cells frequency and phenotype may not be uniform within the tumors of the same subtype. For example heterogeneity has been found within the glioblastoma stem cells population, in which the CD133⁻ population could harbor the same properties of the CD133⁺ cells, which includes self-renewal, and high tumorigenecity [18]. Similar results have been obtained with the breast cancer stem cells using the CD44⁺ surface marker [19].

Furthermore, in series of experiments on melanoma cancer stem cells, Quintana et al. have challenged the frequent assertion that CSCs are a rare population, consequently they demonstrated that in melanoma this population was found in abundance, with CSC's frequency estimated at 1 in 20 cells [20].

The most convincing demonstration of CSC remains the serial transplantation of the cells into animal models. The CSCs should re-establish tumor with the same phenotypic heterogeneity of the parental tumor. A study by Kelly et al. has raised the possibility that cancer stem cells may be a population of selected cells that survive during the xenotransplantation, while the majority of the cells don't survive the foreign environment [21]. However, this hypothesis has been averted considering that tumor cells are more likely to equally receive adequate and sufficient growth factors needed to their growth in the hosting model.

Tumor type	Markers
Acute myeloid, Leukemia	CD34 ⁺ CD38 ⁻
Breast	CD44 ⁺ CD24 ⁻
Breast	ALDH1 ⁺
Brain	CD133 ⁺
Prostate	CD44 ⁺ CD133 ⁺
Head and neck	CD44 ⁺
Colon	CD133 ⁺
Colon	ALDH1 ⁺
Pancreas	ESA ⁺ CD44 ⁺ CD24 ⁺
Pancreas	CD133 ⁺
Lung	CD133 ⁺
Liver	CD90 ⁺
Melanoma	ABC5 ⁺
Ovarian	CD133 ⁺

Table 1. Markers associated with the identification of cancer stem cells in different cancers types.

1.2 Cancer stem cells self-renewal and therapeutics

Several lines of evidence suggest that cancer stem cells are resistant to the conventional radiation and chemo-therapies. Indeed, this population of cells subsists after treatment, while the rest of the tumor bulk cells are eradicated, resulting in re-growth of the tumors, and recurrence of the disease. For instance, glioblastoma stem cells have shown a great resistance to radiation with an enhanced DNA damage response, and more rapid repair of the DNA. *In vitro*, upon radiation of glioblastoma cell lines, and xenograft tumors, the CD133⁺ cells population has been found to be increased, resulting in more tumorigenic cells, and highly aggressive tumors upon serial transplantations [22]. Similarly these cells were found to exhibit resistance to chemotherapeutic agents such as temozolomide, paclitaxel, carboplatin [23]. Similar studies on breast cancers stem cells have showed an increase of the number of CD44⁺/CD24⁻ mamospheres upon treatment as well as a high resistance to radiation. Furthermore, Li et al. demonstrated that the treatment of breast cancer patients with HER2 inhibitor enriched the CSCs population in the tumor biopsies, and were more tumorigenic where injected to mice [24].

The inhibition of cancer stem cells growth relies on identifying and understanding the role of self-renewal pathways in maintaining CSCs. This task may be complicated by the fact that many of the essential pathways are shared with the normal stem cells. Although their roles are not fully understood, cancer stem cells renewal has been shown to be maintained by a plethora of extracellular stimuli, including Sonic hedgehog, Notch, Wnt/ β -catenin, Pten, and the Transforming growth factor- β (TGF- β), pathways, as well as hypoxic condition. Strategies to disturb these pathways have resulted in decrease of the self-renewal and tumorigenicity in several cancer stem cells models [25-27].

For example, it has been demonstrated that Wnt/ β -catenin signaling pathway is essential to maintain the leukemic, breast, and lung cancer stem cells self-renewing potential. Indeed, Wnt signaling knock out using a small interfering RNA decreased from the expression of CSC markers [28]. Pten knockdown enhances breast CSC markers and increase tumorigenicity in a xenograft model. Loss of the Hedgehog results in depletion of the chronic myeloid leukemia CML stem cells [26].

Two of the important signaling cascade in regulation of cancer, the MEK/ MERK (extracellular signal-regulated kinase), and the PI3K/mTOR (mammalian target of rapamycin) pathways have been shown to control the self-renewal and tumorigenicity of glioblastoma stem cells. Inactivation of MEK by a small interfering RNA or a pharmacological inhibitor decreased the self-renewal capacity of glioblastoma stem cells, as well as their tumorigenicity and in addition drove the cells to differentiation [29]. Moreover, Notch blockade by gamma-secretase inhibitors also leads to a decrease of the glioblastoma stem cells tumorigenicity and induces their differentiation [30]. The transforming growth factor- β (TGF β) also influences CSC initiation and maintenance in glioblastoma, and its inhibition decreased from tumorigenicity of the cells [31].

In conclusion, the concept of cancer stem cells as being a small population responsible of maintaining tumor growth has been expanded to many hematological and solid tumor types. Investigating the exact role of this population will help in understanding the genetic bases of cancer development. Furthermore, understanding the programs that govern the self-renewal properties of these cells will help to develop novel therapies targeting the root of the cancer. However, many challenges remain in, including the standardization of more accurate and universal isolation techniques that provide consistent and accurate results.

1.3 Glioblastoma and glioblastoma stem cells

Glioblastoma multiform GBM, a high grade of gliomas, is the most lethal form of brain tumors that is typically resistant to radio and chemotherapies [32]. Glioblastoma stem (GBS) cells have been identified and subsequently isolated and cultured [8, 33]. It has been suggested that these glioblastoma stem cells contribute to the pathogenesis and progression of the disease and importantly, may mediate resistance to various forms of anti-cancer therapies as well as to tumor recurrence [22]. The isolated glioblastoma stem cells were shown to express specific cell surface markers such as the neural stem cell marker and transmembrane glycoprotein prominin (CD133) [8]. Glioblastoma stem cells expressing CD133 are highly tumorigenic. *In vivo* they are highly tumorigenic and alone form characteristic glioblastomas. *In vitro*, glioblastoma stem cells cultured in serum-free medium supplemented with endothelial and basic fibroblast growth factor (FGF) form self-renewing neurospheres. On the other hand and in the presence of fetal bovine serum (FBS), the glioblastoma stem cells are able to differentiate into different lineages expressing glial fibrillary acid protein (GFAP), a glial marker, and neuron-specific class III beta-tubulin (TUJ1). Other markers as the sex determining region Y-box 2 (SOX2), a transcriptional factor in embryonic stem cells [34], has also been shown to identify glioblastoma stem cells.

It is noteworthy that the potential implication of glioblastoma stem cells to the clinical management of glioblastomas has been demonstrated by means of studying the expression of the aforementioned cell surface markers for identification of glioblastoma stem cells. For example, cells co-expressing both the glioblastoma stem cells marker CD133 and proliferation-related ki67 were isolated and cultured from glioblastoma patients treated with surgery followed by adjuvant radiotherapy and target-specific drug treatment [35]. In addition,

the presence or abundance of these glioblastoma stem cell populations correlated with patient poor clinical outcome and response to therapy demonstrating that further interrogation of the biology of these stem cells will favorably impact the clinical management of glioblastoma patients.

Several molecular pathways have been postulated to play important roles in the control and maintenance of glioblastoma stem cells self-renewal and differentiation. Transforming growth factor-beta (TGF- β) by activating the leukemia inhibitory factor (LIF) increases self-renewal of the GBM stem cells and prevents differentiation [31]. In addition, it has also been shown that PTEN blockage and a deletion in the tumor suppressor TP53 increase self-renewal and clonogenicity of the GBM stem cells [30, 36]. However, our knowledge on the molecular pathways governing glioblastoma stem cell self-renewal, survival and differentiation is dismal and limited to a few numbers of studies warranting the need to further study the molecular pathways, e.g. biochemical regulatory processes and pathways, crucial for glioblastoma stemness.

1.4 Mitochondria

Mitochondria are dynamic and plastic cellular organelles carrying out numerous biosynthetic and bioenergetic reactions fundamental to cell function, cell survival but also cell death. These multiple biochemical properties depend on the unique mitochondrial ultra-structural compartmentalization that assures the optimal microenvironment to each reaction. Oxidative phosphorylation is the main biochemical process taking place in mitochondria, a cascade of redox reactions catalyzed by five multi-subunit enzymatic complexes (the respiratory complexes I-IV) and the ATP-synthase leading to the generation of most intracellular ATP[37]. Krebs cycle, heme biosynthesis, β -oxidation of fatty acids, steroidogenesis, part of gluconeogenesis, and amino acid metabolism are only some of the

additional metabolic processes hosted by mitochondria. Although it is evident that mitochondria are crucial for cell fitness and survival, they also regulate cell death via both intrinsic apoptosis and non apoptotic cell death. In fact, under stress conditions if mitochondrial membrane permeabilization occurs the cell is inevitably directed to die following dissipation of mitochondrial transmembrane potential block of ATP synthesis and translocation to the cytosol of activators of caspase-dependent and caspase-independent mechanisms of cell death. Increased anabolism, uncontrolled proliferation and impaired apoptosis are typical characteristics of cancer cells along with sustained angiogenesis and metastatic ability [38]. The first association between mitochondria deregulation and cancer was proposed in 1930 by Otto Warburg who observed an increased rate of aerobic glycolysis in several tumor cells [39]. It is now an emerging concept that mechanisms of neoplastic transformation and tumor progression may early involve mitochondrial dysfunctions.

1.5 Mitochondria biogenesis

Mitochondria biogenesis is an essential process that enables the cells to meet energetic and metabolic demands. It is a complex process that integrates nuclear encoded proteins, lipids imported to the mitochondria from the cytosol, mitochondrial encoded proteins, and replication of the mitochondrial DNA (mtDNA) [40, 41]. The expression of genes necessary to the mitochondrial biogenesis is orchestrated by a network of nuclear DNA-binding transcription factors and coregulators.

To enable the cells meeting their energetic and metabolic demands, mitochondrial biogenesis is regulated in a tissue and signal specific manner [42]. The DNA-binding transcription factors regulate overlapping but distinct classes of mitochondrial genes. The nuclear respiratory factor-1 (NRF1) activates the expression of Oxphos components, mitochondrial transporters, and mitochondrial ribosomal proteins [43]. Furthermore, NRF1 activates mitochondrial transcription factor A (TFAM) thereby, increasing the mtDNA

replication and expression. Upon its activation via phosphorylation or interactions with factors as PGC1, NRF1 translocates into the nucleus and binds to the promoter of different mitochondrial genes [41, 44, 45]. Suppression of NRF1 expression results in a decreased expression of mitochondrial target genes [46]. In addition, NRF1 expression is mainly affected by low energy sensing pathways, AMP-activated protein kinase (AMPK), and the increase in calcium flux in the cells.

Moreover, transcription coregulators allow coordination of physiological external signals, and enhancement of the activity of DNA-binding factors [47]. The PPAR γ coactivator-1 family that includes the PGC1 alpha, PGC1 beta, and PRC promote mitochondrial biogenesis by activating the transcription factors, NRF1 and NRF2 as well as TFAM [48]. The PGC1- α is recruited to the mitochondrial genes target site where they interact with the DNA-binding factors through protein surfaces, thus enabling the recruitment of histone acetyltransferase [49, 50].

PGC1- α senses signals of metabolic and energetic demands of the cells through the interaction with different key factors such as the AMPK and Sirt1 [51]. Both AMPK and Sirt1 are energy sensors that are activated during metabolism stress, such as caloric restriction. AMPK phosphorylate PGC1 enhancing its activity whereas Sirt1 acetylate PGC1 to induce its activity [51-53]. In addition, PGC1 induce their own expression via a regulatory loop seemingly important in enhancing the mitochondrial gene response [54].

1.6 Mitochondrial bioenergetics in cancer cells

In order to sustain and proliferate cells must produce sufficient stores of energy. Most of the adenosine triphosphate (ATP) generated in normal cells is produced *via* oxidative phosphorylation, accounting for 88% of the total energy that is required by the cell [55].

Oxidative phosphorylation (Oxphos) is carried by the electron transport chain (ETC) housed in the inner membrane of mitochondria, which consists of four multi-subunit enzymatic

respiratory complexes. Electrons are carried through these membrane complexes (1 to 4) to molecular oxygen pumping protons across the membrane thus creating electrochemical gradients to phosphorylate ADP, resulting in the production of more than 30 ATPs per glucose molecule.

In contrast, to achieve a growth and proliferation rate larger than the normal cells, malignant cells need to undergo metabolic reprogramming that optimizes the efficiency by which energy is produced and utilized. The first metabolic alteration described in cancer cells was the shift from oxidative phosphorylation to an aerobic glycolysis despite the presence of oxygen -a phenomenon known as the Warburg effect [56]. During aerobic glycolysis, glucose is decomposed to pyruvate, which is converted to lactate and secreted from the cells, resulting in the production of 2 ATPs per molecule of glucose; this low ATP yield is compensated for by a high glucose uptake.

It has been postulated that the increase of the aerobic glycolysis provides to the cells a growth advantage under conditions that are restrictive to normal cells, such as a hypoxic environment and a restricted flow of nutrients [57]. Moreover, the acidic environment created by lactate production enhances tumor invasion and suppresses anticancer immune effectors [58]. Furthermore, it limits the reactive oxygen species ROS, a damaging byproduct of mitochondrial respiration, by metabolizing glucose through the pentose phosphate pathways to generate the nicotidine adenine dinucleotide phosphate NADPH, that ensures a strong antioxidant defense [59]. Moreover, high glucose uptake of the cells can support the biosynthesis by using intermediate of the glycolysis pathway [59].

The conversion from oxidative phosphorylation phenotype to glycolytic phenotype is partly driven by the activation or mutation of oncogenes or tumor suppressor genes, such mechanisms affecting the PI3K/AKT/mTOR axis, c-Myc, AMP-activated protein kinase AMPK, or P53 pathway can control cell metabolism. PI3K/Akt and c-Myc pathways facilitate glycolysis by increasing the glucose uptake. Akt signaling mediates an increase in the glucose flux by

modulating the expression of the glucose transporters Glut1 and Glut4 on the cell surface and enhancing the activity of glycolytic enzymes [60, 61].

Moreover, by a mechanism that still needs to be elucidated, Akt stimulates the association of hexokinases HK1 and HK2, and modulates the expression of phosphofructokinase which catalyzes the initial step of glycolysis [60]. Also through dependent mTOR activation, Akt signaling enhances the protein translation. mTOR coordinates surface expression of amino acid transports, and promotes translation by stimulating ribosomal S6 kinase activity and relieving inhibition of eIF4E [62].

The proto-oncogene c-Myc has been shown to directly alter glucose metabolism by promoting the constitutive elevation of the lactate dehydrogenase LDH-A, that is responsible of maintaining glycolytic flux by converting excess pyruvate to lactate [63]. Moreover, the transcription factor p53 influences the metabolism of cells by favoring the Oxphos to glycolysis. TP53 negatively regulates glycolysis by activating TIGAR, consequently TIGAR degrades to fructose 2-6- biphosphate, an activator of the glycolytic regulator enzyme fructokinase PFK1 [64]. In addition, P53 favors Oxphos by regulating the synthesis of cytochrome c oxidase SCO2 that is required for the assembly of the cytochrome c oxidase mitochondrial complex [65]

In addition, adaptive response to hypoxia stimulates aerobic glycolysis through regulation of the protein and transcriptional activity of the inducible factor-1 (HIF1). HIF-1 targets glucose transporters to increase the glucose uptake, and glycolytic enzymes required to convert pyruvate to lactate including the pyruvate dehydrogenase kinase (PDK1) that limits the entry of glucose to the TCA cycle [66].

Bringing our knowledge of the mitochondria pathways regulation of bioenergetic process as glycolysis, or glutamine metabolism and the cellular signal transduction in cancer cells would make the combination of chemotherapies and targeting mitochondria deregulated pathways very potent for a selective killing of the cancer cells.

1.7 Glutamine metabolism in cancer cells

Glutamine, the most abundant nutrient in circulating blood [67], is another metabolic energy source for tumor cells and was shown to contribute to tumor progression [68]. Glutaminolysis in which glutamine is converted into glutamate then into the Tricarboxylic Acid (TCA) cycle intermediate α -ketoglutarate through the enzymes glutaminase and alanine aminotransferase/glutamate dehydrogenase, was shown to be essential for the growth of tumor cells [69]. Furthermore, xenograft studies have shown that glutaminase expression correlated with tumor growth and inhibition of glutaminase in cells led to inhibition of tumor growth and tumorigenicity [70, 71]. Thompson *et al.* (2007) performed NMR spectroscopic study in glioblastoma cells co-cultured with ^{13}C -labelled glucose and glutamine and demonstrated that glutamine supplied the majority of aneplerotic carbon for the TCA cycle in cells [72].

Glutamate *via* α -ketoglutarate is also the chief source of malate, oxaloacetate and the subsequent NADPH for fatty acid biosynthesis in tumors [72]. Further, glutamine also contributes to maintaining antioxidant pool in cancer cells - the glutamine-derived malate from the TCA cycle serves as the substrate for malic enzyme 1 (ME1) which produces NADPH. In glioblastoma cells the excess NADPH has been shown to contribute towards the synthesis of glutathione (GSH), which is essential to balance the redox demands of tumor cells and microenvironment [72].

Glutamine also impacts cancer metastasis. A recent study by Seyfried *et al.*, showed that inhibition of glutamine metabolism in a mouse breast cancer model using 6-diazo-5-oxo-L-norleucine diminished metastasis of the tumor [73]. Currently, however, there are no extensive reports available on the effect of glutamine on cancer progression in patients. Further work needs to be done to understand the effect of oncogenes like *Akt*, *HIF-1*, *NF- κ B* etc. on the

glutaminase (GAC) enzyme, glutamine metabolism and the “glutamine addiction” characteristic of cancer cells [74].

It is possible to target glutamine metabolism by targeting glutaminase increased activity, the mitochondrial enzyme responsible for hydrolyzing glutamine to glutamate and ammonia providing a therapeutic window through its inhibition [75]. Human B lymphoma and prostate cancer cells demonstrate an increased glutaminase expression dependent on c-Myc [76]. Similarly, MDA-MB231 breast cancer cell line showed higher expression of glutaminase when compared to normal mammary epithelial cells [74]. An inhibitor of Rho GTPas-dependent transformation, known as small molecule 968, inhibited the metabolic enzyme glutaminase and prevented oncogenic transformation.

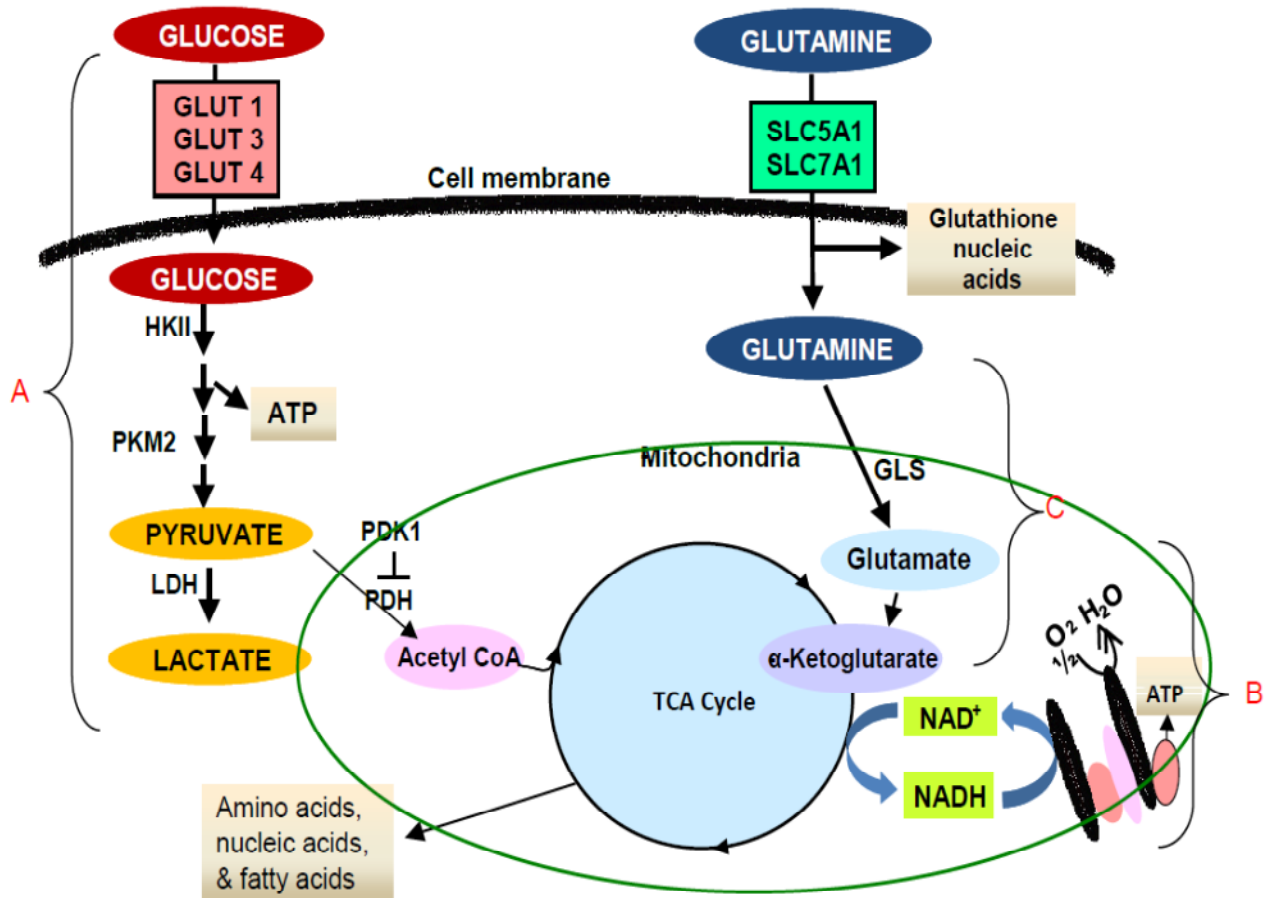


Figure 1. Cancer cell metabolism. Representation of (A) glycolysis, (B) mitochondrial respiration and (C) glutaminolysis during glycolysis cells.

1.8 Mevalonate metabolism

It is the main pathway to generate cholesterol, isoprenoids, dolichol, ubiquinone and isopentenyladenine (Figure 2) [77]. First, hydroxyl methylglutaryl coenzyme A (HMG-CoA) is converted to mevalonate by the HMG-CoA reductase, which is the rate limiting enzyme of the reaction. Then, mevalonate is metabolized to isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). DMAPP and 2 units of IPP are condensed to form farnesyl pyrophosphate (FPP) and geranyl pyrophosphate (GPP). Both GGPP and FPP serve in post translational modification of proteins known as prenylation. Moreover, FPP serves as a precursor for the biosynthesis of cholesterol and dolichols, which serves in N-glycosylation [78].

Mevalonate pathway implication in tumorigenesis was shown by Penn et al. (2009), where ectopic expression of hydroxyl methylglutaryl coenzyme A reductase (HMGCR) increased transformation of the human liver hepatocellular carcinoma cells, HepG2, and immortalized, nontransformed breast cells, MCF7. Furthermore, HepG2 formed bigger and faster growing tumors when injected to severe combined immune-deficient (SCID) mice [79].

Moreover, different studies found that HMGCR levels correlated with bad prognosis and reduced survival. In addition, epidemiological studies showed a decrease risk for breast cancer of 18% in cohort 8106 women taking hydrophobic statin [80].

Statin drugs were developed to inhibit the mevalonate pathway by blocking the rate limiting enzyme HMG-CoA reductase. Statins block cholesterol synthesis, in fact they are used as cholesterol lowering drugs, but they also block the cells from making GGPP and FPP for protein prenylation. Therapeutically, in cancer, statin drugs induced cell death by inhibiting the biosynthesis of GGPP or FPP which are essential to the prenylation of key proteins in prostate epithelial cells, lymphoma, breast cancer cells, glioblastoma, [81-84].

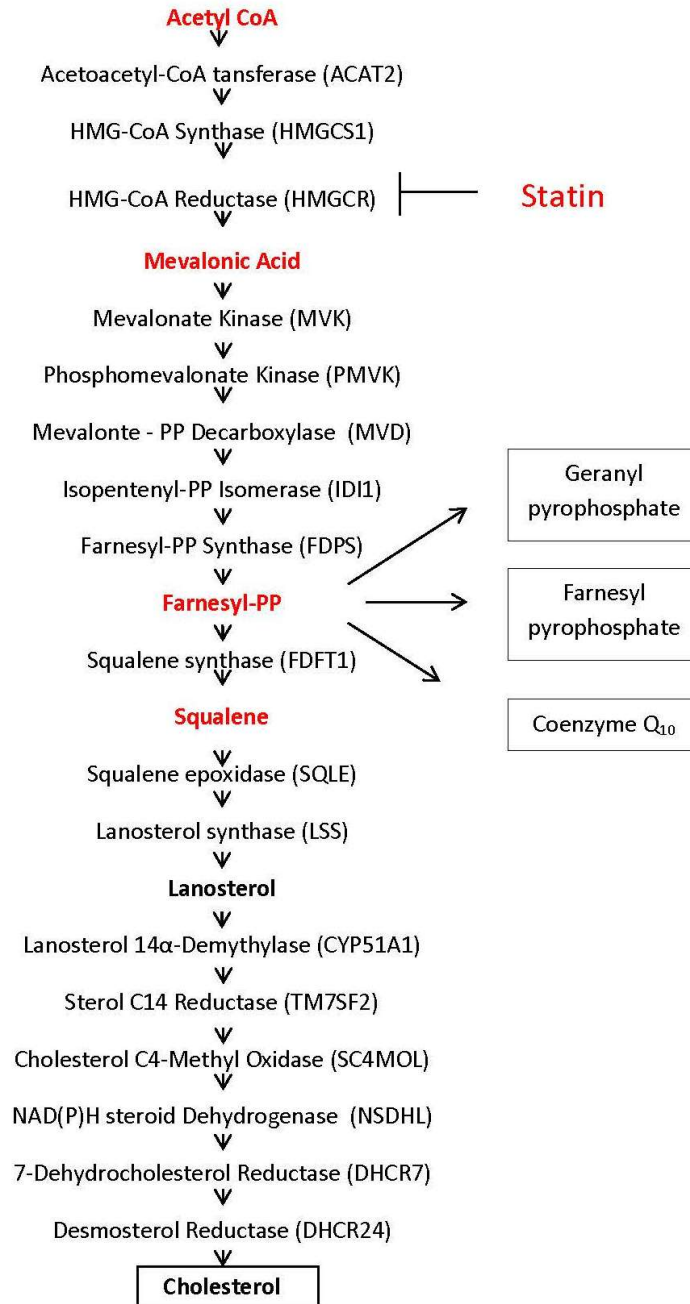


Figure 2. Mevalonate metabolism pathway. Mevalonate pathway produces cholesterol and isoprenoids (geranyl pyrophosphate, farnesyl pyrophosphate) which are important for protein prenylation. Statin inhibit the mevalonate pathway at the HMGCR level.

1.9 Hypothesis and specific aims

Glioblastoma multiform is the most lethal form of brain tumors that is typically resistant to various forms of therapies. Recently, it has been suggested that glioblastoma stem cells contribute to the pathogenesis of the disease and may mediate tumor recurrence and resistance to anti-cancer therapies. The proposed project aims to explore the bioenergetics pathways, typically derailed in malignant compared to normal cells, in glioblastoma stem cells in an effort to increase our understanding of the biology of this unique cell population.

I **hypothesize** that glioblastoma stem cells have a lower mitochondrial respiration, and rely on glutamine metabolism and mevalonate pathway for cell proliferation and survival.

The **major goals** of this study were to compare mitochondria respiration and energy production, glutamine metabolism, and mevalonate pathway in glioblastoma stem cells and FBS induced cells. Specifically to understand the role of glutamine in GBM stem cells and to identify a new metabolic pathway to be targeted to eliminate GBM stem cells.

The **significance** of this study relies on the importance of glioblastoma stem in the pathogenesis of glioblastoma and their resistance to therapies. The identification of deregulated metabolic pathways, such as increased mevalonate genes expression, in the stem cells compared to differentiated cells, may facilitate the specific targeting of glioblastoma stem cells for the goal of more efficacious clinical management of the morbid glioblastoma disease. In order to test my hypothesis, my specific aims were:

Specific Aim1: Characterize the bioenergetics of glioblastoma stem cells and differentiated cells.

Specific Aim2: Determine the effect of blocking glutaminolysis on glioblastoma stem cells.

Specific Aim3: Determine the therapeutic effect of mevalonate pathway blockade in glioblastoma stem cells.

CHAPTER 2. Material and methods

Cell culture

In this work we used glioblastoma stem cells that have been identified and isolated from fresh surgical specimens of glioblastoma multiform at the University of Texas MD Cancer Center, department of Neurosurgery. GSC11 and GSC23 GBM stem cells are typically cultured in medium devoid of serum but containing endothelial and basic fibroblast growth factors supplementation. To induce differentiation, cells will be incubated in culture medium containing 5% FBS. These cells were previously characterized as glioblastoma stem cells by their high tumorigenicity as well as their expression of the marker CD133 [85]. In contrast and in the presence of FBS, the stem cells differentiate to express GFAP and BIII tubulin. Therefore, incubation of glioblastoma stem cells in serum-containing medium appears to be a reliable method for differentiation induction and may be exploited to study mechanisms governing differentiation of these stem cells.

Reagents and chemicals

GSC11 and GSC23 cells were maintained in MDM/F12 (Cellgro, Mediatech, Inc.) supplemented with B27 (17504-044, Life technologies), 20 ng/ml of epidermal growth factor (EGF), and 10 ng of basic fibroblast growth factor (bFGF) (Miltenyl Biotech, Bergisch Gladbach, Germany). Differentiated cells were kept in similar medium with fetal bovine serum (Sigma-Aldrich , St. Louis, MO).

Glutaminase inhibitor, 5-(3-Bromo-4-(dimethylamino)phenyl)-2,2-dimethyl-2,3,5,6 tetrahydrobenz[a]phenanthridin-4(1H)-one (Compound 968, C968) and betulin were purchased from EMD Millipore (Billerica, MA) and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and 3 mg/ml, respectively.

Simvastatin, geranylgeranyl phosphatase ammonium salt, farnesyl pyrophosphate and squalene were obtained from Sigma-Aldrich (St.Louis, MO).

Oxygen consumption

To measure oxygen consumption 3×10^6 cells are re-suspended in 1ml of fresh culture medium pre-equilibrated with 21% oxygen, and then placed in a sealed respiration chamber equipped with a thermostat control and a micro-stirring device (Oxytherm, Hansatech Instrument, England). Oxygen consumption was measured at 37 °C with the Clark-type oxygen electrode disc, using the conditions recommended by the manufacturer. Respiration was expressed as nanomoles of O₂ consumed as a function of time in minutes [86] .

Measurement of ATP

Cellular ATP was measured using the CellTiter-Glo Luminescent Cell viability Assay (Promega Biosciences, California). Cells were plated at 25,000 cells in 100 µl media per well, in a 96 well-plates. 100 µl cellTiter-Glo is added per well, and plates were incubated for 10 minutes at room temperature on an orbital shaker. Luminescence was measured using a Fluoroskan luminescence scanner (Thermo Scientific, Waltham, MA). ATP level in the cells was evaluated by the luminescence intensity generated by the luciferine catalyzed by luciferase in presence of Mg⁺², ATP and molecular oxygen.

Cell viability assay (MTS)

Cells were plated at a density of 5,000 cells per well in 96-well plates. The cells were then treated with different concentrations of C968 and incubated for the appropriate amount of time. Following which, 40 µL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) (Promega, Madison, WI) was added to each well and

incubated at 37 °C (5% CO₂) for 3 hours. The absorbance was measured at 492 nm using a Multiskan plate reader (Thermo Scientific, Waltham, MA).

Western blot

Cells were collected by centrifugation, and cell pellets were washed with iced-cold PBS and lysed for 30 minutes on ice with ice-cold RIPA buffer (50 mM Tris-HCL pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with Protease inhibitor cocktail (05892970001, Roche, Indianapolis, IN) and a phosphatase inhibitor cocktail (04906837001, Roche, Indianapolis, IN). Protein concentrations were measured using the ABC protein assay (23225, Thermo Scientific, Rockford, IL) following the manufacturer's instruction. 20-25 µg of proteins were separated by electrophoresis on SDS-PAGE, transferred to a nitrocellulose membrane and then blotted with specific primary antibodies followed by a secondary antibody.

CD133 extracellular staining

Neurospheres were disassociated into single cells with accutase (Sigma-Aldrich), and then washed. 3×10^5 cells were re-suspended in 80 µL of MACS buffer (Miltenyl Biotech, Bergisch Gladbach, Germany) and 20 µl FcR blocking reagent (130-059-901, Miltenyl Biotech, Bergisch Gladbach, Germany). 3 µL isotope control, mouse IgG1/APC (130-090-845, Miltenyl Biotech, Bergisch Gladbach, Germany), was added or 5µl of CD133/2 (clone293C3)-APC, Human (130-090-854, Miltenyl Biotech, Bergisch Gladbach, Germany) and incubated at 4°C for 15 minutes. Cells were spun down, washed and re-suspended in 200 µL PBS for fluorescence measurement using FACScalibur flow cytometry (Becton Dickinson, San Jose, CA) equipped with CellQuest Pro Software. The isotope control was used to establish a gate in the APC channel. Cells showing signal beyond the gate were considerate to be CD133-positive.

Mitochondrial membrane potential measurement

Mitochondrial membrane potential was assessed by incubating 5×10^5 cells for one hour with 1 μ M of Rhodamine 123 (Invitrogen), a cell-permeant, cationic, fluorescent dye that is readily sequestered by active mitochondria without cytotoxic effects. Cells were washed and resuspended in PBS for flow analysis using a FACScan flow cytometer (Becton Dickinson Bioscience, San Jose, CA) with the CellQuest software. A decrease in Rhodamine 123 capture reflects a decreased mitochondrial potential.

Mitochondrial mass measurement

Mitochondrial mass was measured using MitoTracker Green (Invitrogen, Carlsbad, CA). Cells were incubated for one hour with 60 nM MitoTracker Green, which efficiently stains mitochondria. MitoTracker Green staining was quantified by flow cytometric analysis for GBM stem cells and differentiated counterparts.

Glutamine uptake

To measure glutamine uptake, culture media was replaced by a glutamine-free DMEM/F-12 and incubated for 4 hours. Then [14 C]L-glutamine (0.05 μ Ci/ml) (Perkin Elmer, Waltham, MA) was added and incubated at 37 °C for 30 minutes. Cells were collected by centrifugation, washed with PBS and lysed with 0.2% SDS/0.2N NaOH solution, and incubated for 1 hour. Radioactivity in the cell lysate was quantified by beta scintillation counter (Beckman).

Cell proliferation

To test cell proliferation, an equal number of cells were plated in 1ml of medium in 12 well-plates. Cells were counted every few days. The day of the counting, neurospheres are dissociated, and resuspended in 1ml of PBS to be counted with an automatic counter.

Soft agar assay

Cells were dissociated and seeded in a 6 wells plate at a density of 10,000 cells per well in a stem cells medium with 0.33% agar. Bottom layer consisted of a 0.7% agar in stem cell medium. Plates were incubated for 4 weeks, and then stained with MTT to visualize colonies.

Real time PCR

Total RNA was extracted from glioblastoma stem cells in serum-free medium and cells in medium containing 5% FBS at 24, 72 hours and 7 days. Total RNA was isolated, along with elimination of genomic DNA, using the RNeasy kit from Qiagen (Qiagen, Valencia, CA). cDNA was synthesized from 5 µg RNA. PCR was carried with Syber Green PCR Mix (4344463, Applied Biosystems, Grand Island, NY) on the Viiia Real-Time PCR System (life Technologies, Grand Island, NY). Primers used are found in table 2.

Table 2. Human PCR primer sequences

Gene	Forward	Revers
Olig2	TAGAACTGTGGCCGTTTCCTC	TGAGTCGGTGGGGTAGTTTC
SOX2	AACCCCAAGATGCACAACCTC	CGGGGCCGGTATTTATAATC
CD133	CTAGCCTGCGGTCATCTCTC	GGATTGATAGCCCTGTTGGA
TFAM	CCGAGGTGGTTTTTCATCTGT	AGTCTTCAGCTTTTCCTGCG
PGC1-α	ACTCAGCAGCAGTTGACCCT	GGACTAGGAGAAGCAGGGCT
COX2	TATCACCTTTCATGATCACGC	GACGATGGGCATGAAACTG
20KDa	ACATCCCCCACACCTGTTTC	ATAGTGAACCACCCGCTCTG
ND4	GACTCCCTAAAGCCCATGTCTG	TTGATCAGGAGAACGTGGTTAC
Cyt b	CGCCTACACAATTCTCCGATC	CGGGTGTTGATGGGTGAGTC
ATPase	CTGTTTCGCTTCATTCATTGCC	GTGGCGCTTCCAATTAGGTG
HMGCS1 [87]	GGGCAGGGCATTATTAGGCTAT	TTAGGTTGTCAGCCTCTATGTTGAA
HMGCR	GGCCCAGTTGTGCGTCTT	CGAGCCAGGCTTTCATTCT
MVK [87]	TGGACCTCAGCTTACCCAACA	GACTGAAGCCTGGCCACATC
MVD [87]	TGAACTCCGCGTGCTCATC	CGGTAAGGCGGAGATTATC
LSS [87]	TGCAGAAGGCTCATGAGTTCCT	TCTGGTAGTCGGGAGGGTTATC
SQLE [87]	CGTGCTCCTCTTGGTACCTCAT	CGGTCAAGGCGGAGATTATC
SREBP1	GTGGCGGCTGCATTGAGAGTGAG	AGGTACCCGAGGGCATCCGAGAAT
SREBP2	GCAGATGGGCAGCAGAGTTCC	GTGGTCAGGAGGCGGCAATG

CHAPTER 3. Metabolic state of glioblastoma stem cells and FBS induced cells

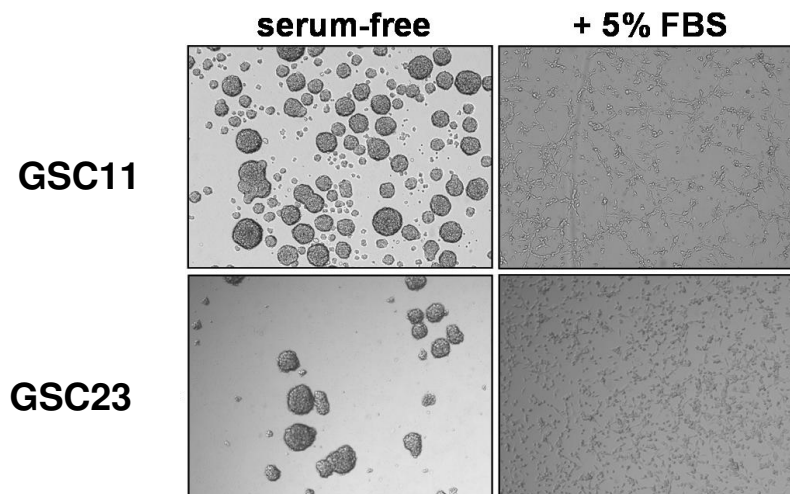
3.1 Results

GSC23 and GSC11 express stem cells markers which are decreased with the induction of differentiation with FBS

In this study we used two human glioblastoma stem cell lines: GSC23 and GSC11, which were previously derived from surgically removed tumors. Glioblastoma stem cells are cultured in serum free medium supplemented with EGF and bFGF and grow in the form of neurospheres (Figure 3A). Moreover, the induction of differentiation by adding 5% fetal bovine serum (FBS) resulted in the loss of neurospheres forming capacity and the attachment of the cells to the culture plates (Figure3A).

To ascertain whether GSC23 and GSC11 cells display stem cells features, we assessed for the expression of the stem cell markers: CD133, Olig2 and SOX2 by quantitative real time PCR. We found that both GSC23 and GSC11 cells expressed these markers; furthermore we observed a decrease of stem cells marker mRNA levels when the cells were induced to differentiate with FBS (Figure 3 B-C).

A



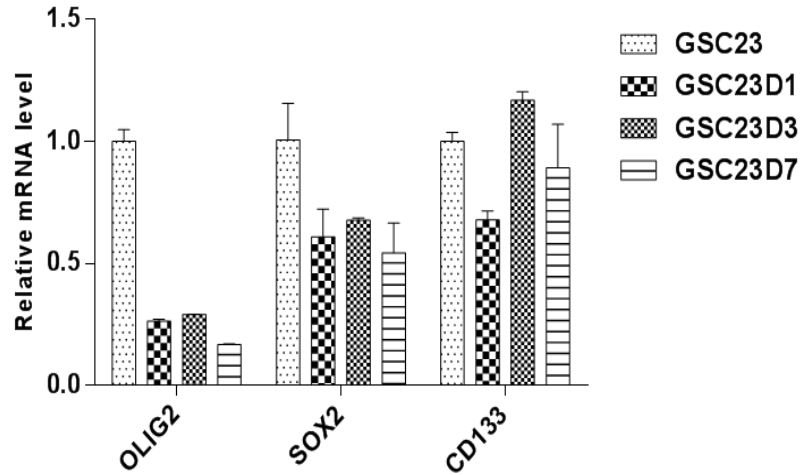
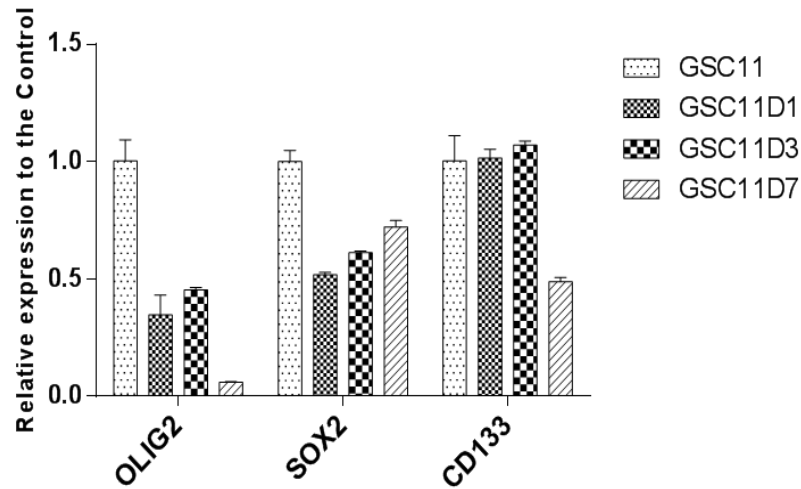
B**C**

Figure 3. Differentiation of glioblastoma stem cells. A, GSC11 (top row) and GSC23 (bottom row) cells form the typical neurospheres when incubated in serum-free medium (left column), and changed morphology after 3 days when 5% FBS was added (right column). Cells were observed under an inverted light microscope.

B-C, mRNA expression of GBM stem cells markers Olig2, SOX2 and CD133. GSC23 and GSC11 cells were cultured in serum free medium or in medium containing 5% FBS for 1, 3 or 7 days, after which total RNA was isolated, and gene expression was assessed by quantitative real time PCR.

Glioblastoma stem cells exhibit reduced mitochondrial respiration which increases upon exposure to fetal bovine serum.

As mentioned before, we hypothesized that glioblastoma stem cells exhibit reduced mitochondrial bioenergetics and respiration compared to their differentiated counterparts. We sought to assess oxygen consumption, as an indication of mitochondrial respiration, in glioblastoma stem cells cultured in serum-free medium as well as in stem cells incubated in medium containing 5% FBS. Towards this goal, GSC23 and GSC11 cells were made to differentiate by exposure to 5% FBS for 72 hours. Oxygen consumption was then measured at 37°C with a Clark-type oxygen electrode disc. Exposure of GSC11 and GSC23 glioblastoma stem cells to 5% FBS for 72 hours, significantly increased oxygen consumption rate (* $p < 0.05$). The rate increased by approximately 2.6-fold and 6.2-fold in the GSC11 and GSC23 glioblastoma stem cells exposed to FBS, respectively (Figure 4). These findings suggest that an increase in mitochondrial activity is coupled to differentiation induction. We seek to further expand these results to increase our understanding of the role of mitochondrial bioenergetics in glioblastoma stem cell differentiation.

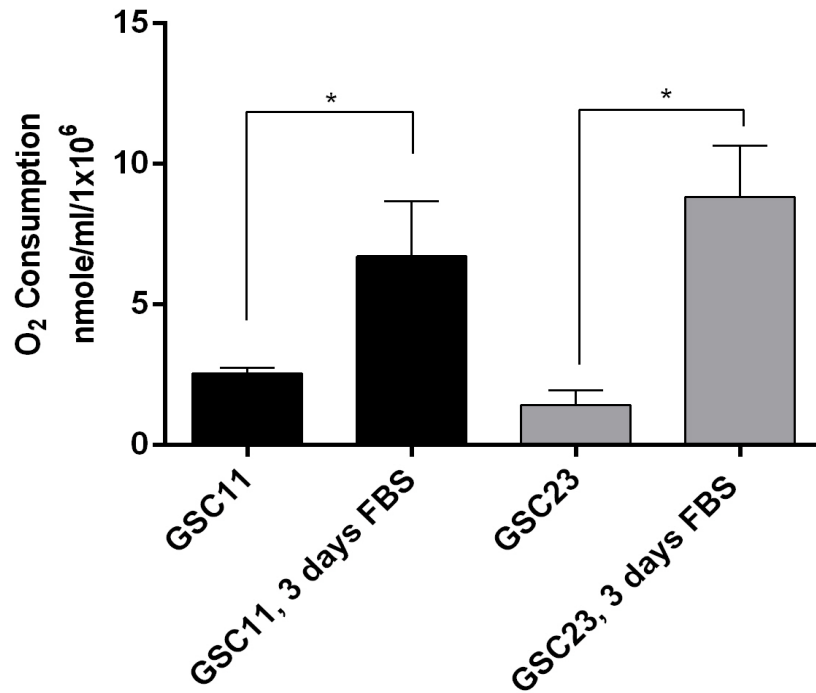
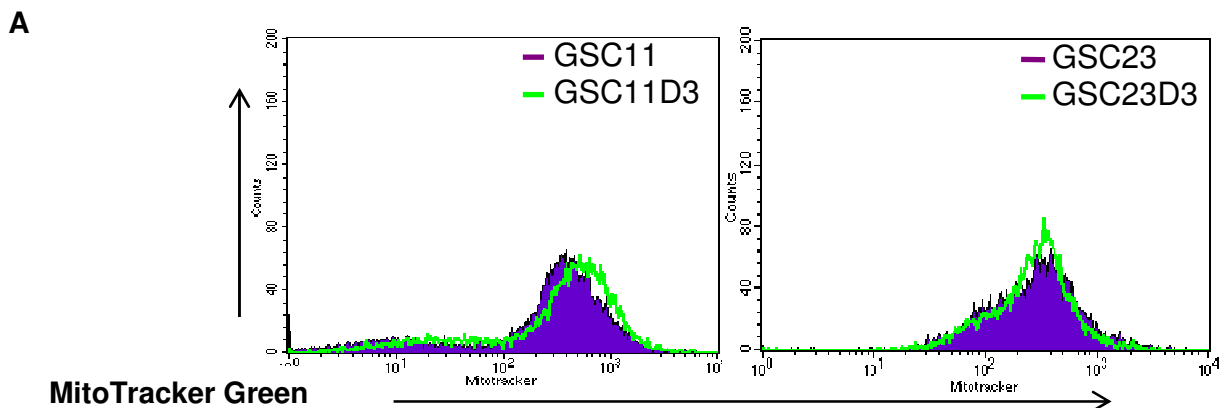


Figure 4. Increase in oxygen in glioblastoma stem cells following exposure to FBS. GSC11 and GSC23 glioblastoma stem cells were resuspended (3×10^6) in fresh culture medium equilibrated with 21% oxygen. Concurrently, the cells were also incubated in medium containing 5% FBS. After 72 hours, oxygen consumption rate was assessed and expressed as nanomoles of oxygen consumed as a function of time in minutes. Consumption rate was calculated from three independent experiments. P-values indicating statistical significance (*, $p < 0.05$) in differences of oxygen consumption rate were calculated by the Student's t-test.

No increase in mitochondria mass or biogenesis upon differentiation

Because we observed that there was an increase in the mitochondrial respiration upon exposing GBM stem cells to FBS, we hypothesized that there might be an increase in the mitochondrial mass to support mitochondrial respiration. Therefore, we measured the mitochondrial mass in GSC23 and GSC11 cells in comparison to cells exposed to FBS for 3 days by incubating the cells with the mitoTracker Green dye for one hour, followed by flow cytometric analysis. However, we didn't observe a significant difference in mitochondria mass in the FBS induced cells compared to the GBM stem cells (Figure 5A).

We further sought to explore mitochondrial bioenergetics as a mean to support the increase of the mitochondria respiration in FBS induced cells. As explained in the introduction, mitochondria have their own genome which carries many of the genes encoding for proteins needed for the respiratory chain. In addition, mitochondria genome transcription involves nuclear encoded transcription factors as TFAM, and PGC1- α . To assess if there was an increase of the transcription of the mitochondrial genome, we measured the mRNA levels of mitochondria encoded genes COX2, 20kDa, ND4, Cytb, ATPase and the transcription factors TFAM and PGC1- α in GSC23 and GSC11 stem cells and FBS induced counterpart for 1 and 3 days. We didn't observe any significant difference between GBM stem cells and differentiated cells to support a potential increase in mitochondria (Figure 5B).



B

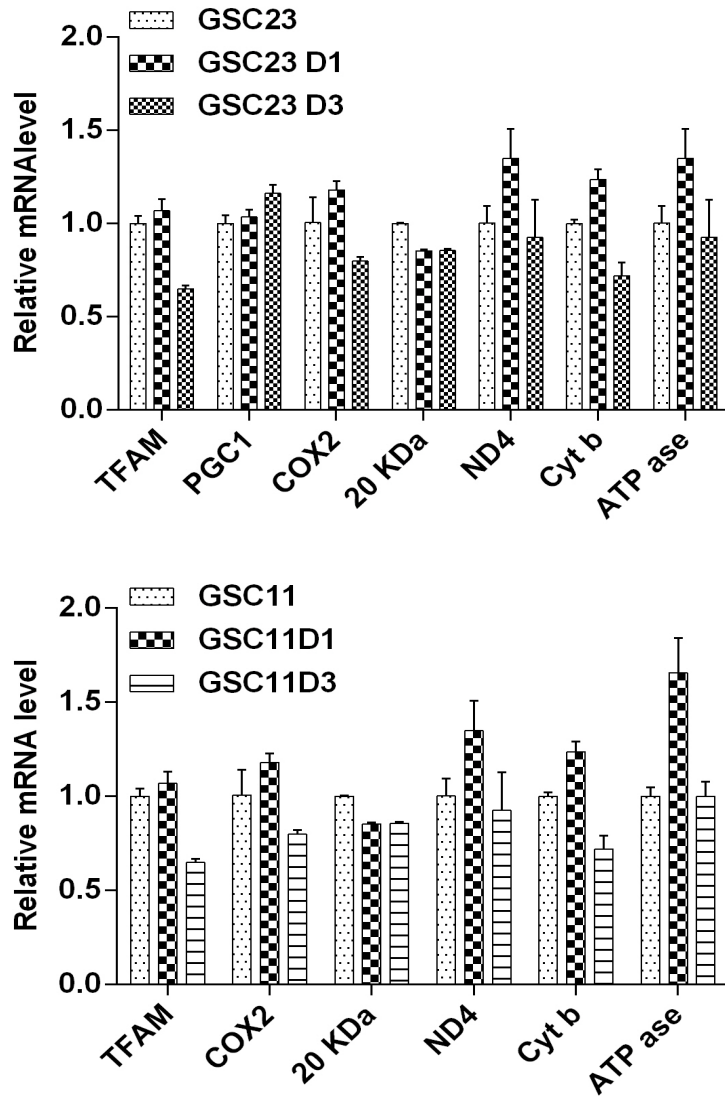


Figure 5. FBS does not induce an increase in mitochondrial mass or mitochondria biogenesis. A, GSC 23 (left) (colored filled) cells were induced to differentiate with FBS for 3 days (no fill histogram). Mitochondria mass was assessed by flow cytometry and no significant increase of the mass was observed. Similarly, GSC11 (right) was induced to differentiate (no fill), after which mitochondria mass was assessed by flow cytometry.

B, GSC 23 (top graph) and GSC11 (bottom graph) were induced to differentiate with FBS. RNA was isolated and transcripts levels were assessed by QRT-PCR. Results show no significant difference in the transcripts after FBS differentiation.

Effect of metabolic inhibitors on ATP levels

We found that mitochondrial respiration increased during induction of differentiation with FBS, therefore we speculated that there might be a shift in the ATP generation from glycolysis to oxidative phosphorylation with differentiation. We induced GSC23 to differentiate with FBS, for 3 or 7 days. After which the cells were dissociated and re-plated at a density of 25,000 cells per well in 96 well-plates. The cells were then treated with metabolic inhibitors for 6 hours. 2 deoxyglucose (2-DG) was used to inhibit glycolysis (2.5-10 μM), Antimycin A (2-20 nM) was used to inhibit mitochondria respiration. After 6 hours of incubation, ATP production was assessed. We observed that with differentiation (GDC23D7) ATP production inhibition was more sensitive to antimycin A, which correlates with the increase of the mitochondria respiration (Figure 6).

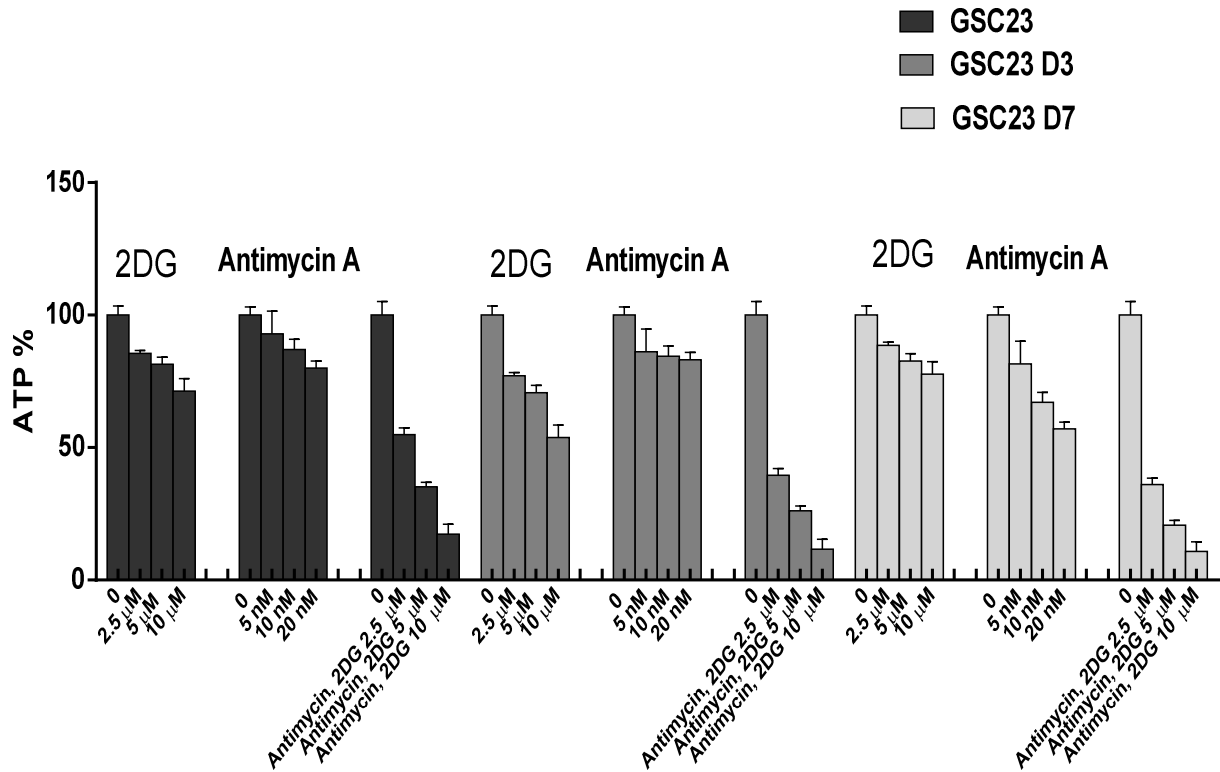


Figure 6. Differentiated GBM stem cells are more sensitive to antimycin A. GSC23 were induced to differentiated with FBS for 3 and 7 days, after which the cells were dissociated and replated at 25,000 cells per well in a 96 well-plates. Cells were treated with 2-deoxyglucose (2DG), Antimycin A, or a combination of the two. Cells were incubated for 6 hours at 37°C, and 100 μ l of stop and glow was added to each well and left to shake for 10 minutes. Luminisence was assessed.

Molecular changes are observed upon differentiation with FBS

To understand the molecular mechanism behind the increase of mitochondrial respiration, we compared the protein expression of pyruvate dehydrogenase kinase (PDK1) and glutaminase (GLS2). Pyruvate dehydrogenase kinase (PDK1) inactivates the pyruvate dehydrogenase (PDH), the enzyme converting pyruvate to acetyl-CoA, by its phosphorylation. This suggests that the tricarboxylic acid cycle (TCA cycle) might be slowed down by limiting the conversion of the pyruvate to acetyl-CoA. Therefore, we assessed for another enzyme (GLS2) that could supply the TCA cycle with α -ketoglutarate, which could have increased the mitochondrial respiration. We found that the protein expression of GLS2 was increased with FBS, suggesting that glutamine might be used by the cells as a substrate to fuel the TCA cycle.

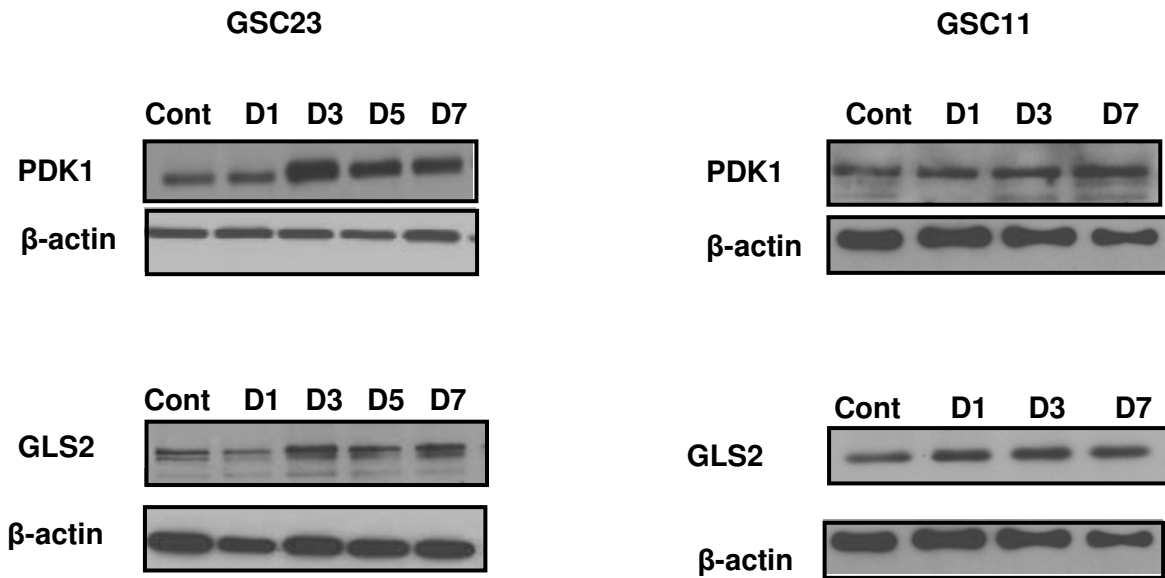


Figure 7. Exposure of glioblastoma stem cells to FBS increases the protein expression of PDK1 and GLS2. GSC23 and GSC11 glioblastoma stem cells were incubated in medium containing 5% FBS for 1 (D1), 3 (D3), or 7 (D7) days. Total protein lysates were prepared from the cells in FBS as well as from cells cultured in serum-free medium (Cont). Lysates (20 µg) were subjected to western blotting to assess protein expression of the PDK1 and GLS2.

3.2 Discussion

Energy metabolism in the form of Adenosine triphosphate (ATP) is generated intracellularly either by glycolysis or mitochondrial oxidative phosphorylation [88]. Metabolic reprogramming is required for cell homeostasis and survival as well as for the sustained proliferation of cancer cells. Furthermore, cancer cells frequently exhibit increased glycolysis and depend largely on this metabolic pathway for generation of ATP to meet their energy needs. As mentioned before, it is now being increasingly recognized that cancer stem cells play crucial roles in the survival and clinical relapse of various malignancies [89]. However, metabolic pathways and energetics in cancer stem cells are still poorly understood. We sought to understand mitochondrial bioenergetics in glioblastoma cells that had been previously isolated from patients (GSC23 and GSC11).

We found that GBM stem cells GSC23 and GSC11 cells cultured in serum free medium expressed cancer stem cells marker Olig2, SOX2, CD133, which were downregulated upon induction of differentiation with fetal bovine serum (FBS). These cells can serve as a good system to evaluate mitochondrial bioenergetics in GBM stem cells and differentiated cells.

We found that glioblastoma stem cells exhibited low mitochondrial respiration. Moreover, mitochondrial respiration in the cancer stem cells increased when induced to differentiate with FBS (Figure 4). It is well appreciated that a switch to active mitochondrial respiration requires the acquisition of properties such as an increase in the number of mitochondria (mitochondrial mass), increased function of the mitochondrial respiratory chain and expression of its complexes subunits and the expression of biogenesis markers. However, in our study we didn't observe any change in the mitochondria biogenesis. Therefore, we can suspect that there was an increase of mitochondria function, which remains to be tested.

Furthermore, previous study by Marin-Valencia et al., investigating brain tumor metabolism *in vivo* in an orthotopic mouse model of primary human glioblastoma, revealed that brain tumor

cells avidly consumed glucose to utilize it to fuel energy through oxidative respiration [90]. Those findings were contradictory to what it has been previously observed in GBM cell lines, where the cells were more glycolytic. However, it supports our finding that more differentiated cells (GBM stem cells induced with FBS) had a higher mitochondrial respiration compared to GBM stem cells, which from a previous study of our group, were shown to be more glycolytic [91].

Also, it was suggested that the differences in the metabolic profiles and pathways activation among various types of tumors are in part due to the tumors unique microenvironments. The different tumor microenvironments in which the cells are subject to differential fluctuation in oxygen levels and changes in the nutrient abundance, tailor the energetic remodeling and metabolic profiles for optimal survival of the specific cancer cells [92]. In a review by Jezek *et al*, it was postulated that metabolic alterations follow a “wave of gene reprogramming”, where at first aerobic glycolysis during a lack of oxygen is enhanced due to the activation of hypoxia-inducing factor 1 (HIF1 α) transcriptional factor which in turn transactivates the expression of various glycolytic enzymes [93]. However, when energy demands exceed nutrients supplied by the blood, the tumor cells typically re-establish mitochondrial respiration to meet the energy levels and demands needed to maintain the increased proliferation of the cells. For example, mesenchymal stem cells were shown to be highly glycolytic but shift to oxidative phosphorylation during their differentiation into fibroblasts [94]. Moreover, hypoxic niche was identified as critical regulator of stemness of GMB stem cells. This might support our finding for a low mitochondrial respiration in GBM stem cells. Interestingly, GBM stem cells GSC23 and GSC23 were shown to have enhanced stem cells properties under lower O₂ tension [85].

Our findings that respiration is low in GBM Stem cells and increase with differentiation support the hypothesis where cancer stem cells are to be found in specific niches.

Furthermore, we found that pyruvate dehydrogenase was elevated following differentiation, this might slow the entrance of pyruvate into the TCA cycle, however, the increase of GLS2 might provide a substrate, α -ketoglutarate, to fuel TCA cycle and increase mitochondrial respiration. These findings provide a molecular basis to the elevation of respiration with differentiation, however more functional assays must be conducted in the future.

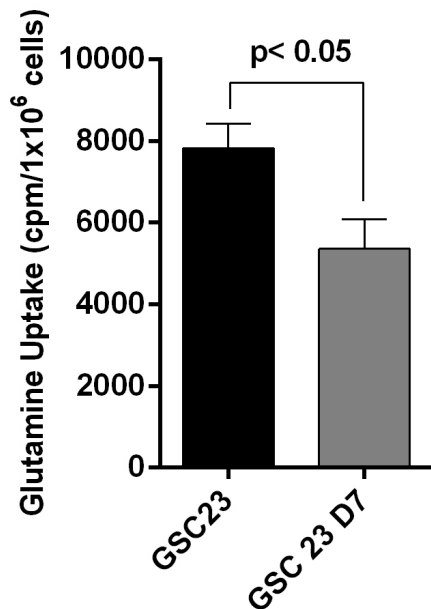
CHAPTER 4. Glutamine role in glioblastoma stem cells maintenance

4.1 Results

Glutamine consumption in cancer stem cells and differentiated cells

Glutamine is an important amino acid in many cancer types; it has been implicated in transformation, proliferation and survival. We sought to explore glutamine metabolism in glioblastoma stem cells and differentiated cells. To assess glutamine metabolism in GBM stem cells, we first measured glutamine uptake in GBM-SC and differentiated cells. Strikingly, GSC23 and GSC11 GBM stem cells consumed two folds greater of glutamine than their differentiated cells counterparts (GSC11D7, GSC23D7), which were induced with 5% FBS for 7 days ($p < 0.05$) (Figure 8).

A



B

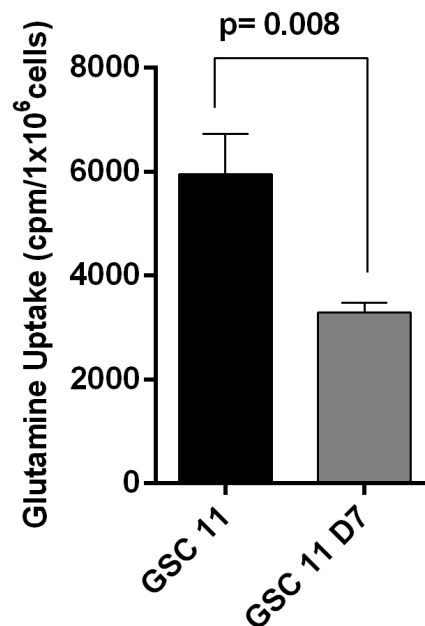


Figure 8. Comparison of glutamine uptake between glioblastoma stem cells and differentiated cells. A, GSC23 were maintained in stem cells media, and GSC23D7 were induced to differentiate with FBS for 7 days. 1×10^6 cells were replated in glutamine free DMEM/F12 and incubated for 4 hours. [^{14}C]- Glutamine (0.5 $\mu\text{Ci/ml}$) was added to the cells and incubated for 30 minutes. Cells were then collected, spun down and washed twice with cold PBS. Cell pellets were re-suspended in 200 μl lysis buffer and incubated at 37°C for one hour. Uptake of the labeled glutamine was quantified by scintillation counting of the cellular lysate. Data represent the mean \pm SD of biological triplicate samples.

B, glutamine uptake was measured in GSC11 cells and GSC11 induced with FBS for 7 days (D7), 1×10^6 cells were used. Data represent the mean \pm SD of biological triplicate samples.

Glutamine affects cell proliferation but does not cause cell death

The finding that GBM stem cells consumed more glutamine, prompted us to hypothesize that glutamine might be mediating proliferation and survival of GBM stem cells. Subsequently, to investigate the role of glutamine, we blocked glutaminolysis by employing a small molecular weight inhibitor, C968. C968 was shown to block glutaminase activity at concentration of 1-10 μM [74]. A 7 days treatment of GSC23 cells with C968 (10 μM) resulted in the formation of smaller neurospheres as compared to control DMSO treated cells, as observed under the phase contrast microscopy picture (Figure 9).

Next, to determine if the smaller neurospheres resulted from cell death, we examined the effect of C968 on cells survival. GSC23 and GSC11 cells were treated with increasing concentrations of C968 (1 to 20 μM), for 3 or 5 days. We analyzed the cytotoxicity of C968 on the cells by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS). Glutaminolysis inhibition was not cytotoxic to the cells (Figure 10).

Furthermore, we examined cell proliferation as a cause to the formation of smaller neurospheres. An equal number of GSC11 cells and an equal number of GSC23 cells were cultured in the absence or presence of C968 (10 μM). Cells were counted every few days. At 3 days the difference in proliferation of GSC23 wasn't significant, however, after 7 days the cells treated with C968 were significantly proliferating slower than the untreated cells and continued to grow significantly slower at 10 days. Similarly, GSC11 cells showed a significant difference in cells number at 7 days and 10 days, however, it didn't show any difference at 3 days, which can be explained by their slower doubling time and time to recover after dissociation with accutase before plating (Figure 11). These data indicate that proliferation and not survival was affected by blocking glutaminolysis with C968.

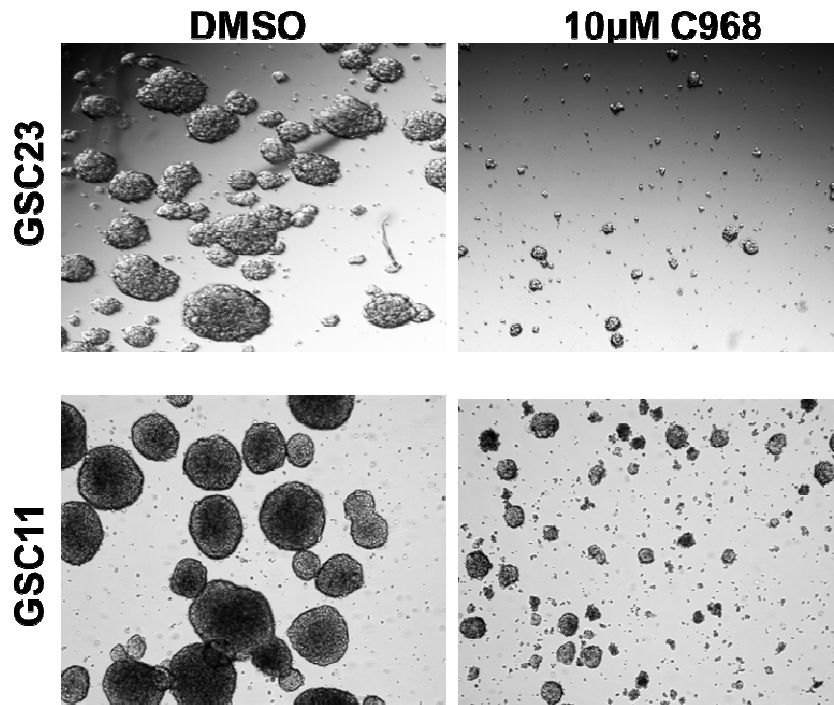
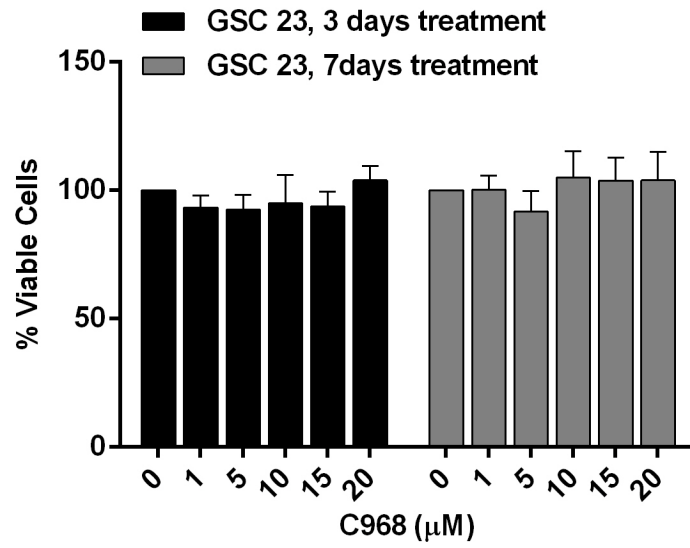


Figure 9. C968 treatment results in the formation of smaller neurospheres. GSC23 cells (top) were dissociated into single cells. An equal cell number was then plated and treated with either DMSO (left) or 10 μ M C968 (right) and cultured for 7 days. Also GSC11 were dissociated into single cells and plated with DMSO (left) or 10 μ M of C968 for 7 days (right). Neurospheres were observed under light microscopy at magnification x40.

A



B

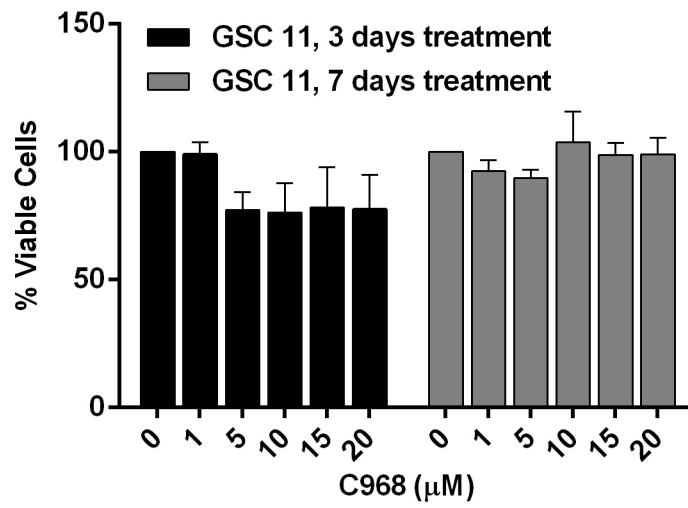
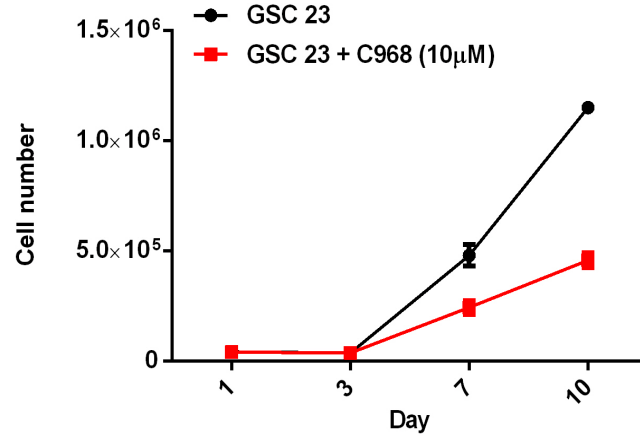


Figure 10. GBM stem cells viability is unaffected by C968. (A) GSC23 neurospheres and (B) GSC11 neurospheres were disassociated into single cells and seeded in 96 well-plates at a density of 5,000 cells per well. Cells were treated with either DMSO, (0 μ M), or C968 at doses ranging from 1-20 μ M for 3 or 7 days, after which MTS was added, and incubated for 3 hours at 37°C. Measurement was done at 432nm and cell viability was normalized to control DMSO treated cells. Results are represented as mean \pm SD of three independent experiments.

A



B

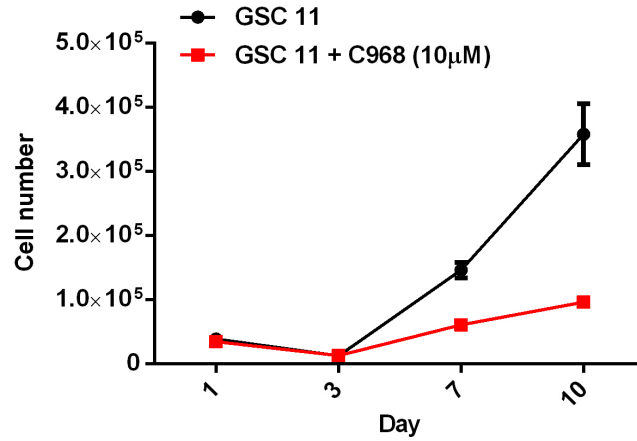


Figure 11. C968 slows GBM stem cells proliferation. GSC23 (A) and GS11 (B)

neurospheres were dissociated, seeded equally in 12 well-plates and left untreated or treated with 10 µM C968. Biological triplicate were counted at the indicated times. Data points represent the mean value ± SD.

Effect of C968 on CD133 glioblastoma stem cells marker

To further characterize the role of glutaminolysis in GBM Stem cells, we explored if the inhibition of glutaminolysis affected the stemness of the cells. As previously shown, GBM stem cells express the stem cell marker CD133, thus we determined the expression of CD133 after treatment with C968.

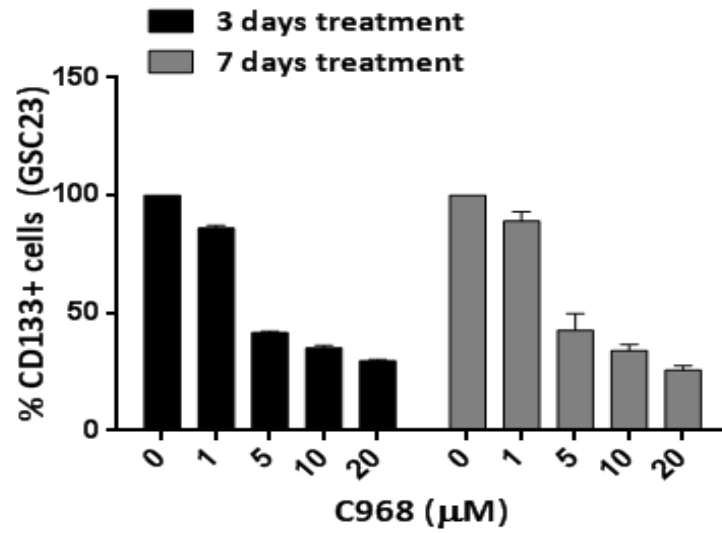
GSC23 and GSC11 cells were treated with various concentrations of C968 (1-20 μ M), for either 3 or 7 days. Flow cytometric analysis showed a suppression of the CD133 in a dose dependent manner (Figure 12 A-B). At 10 μ M of C968 there was a suppression of about 70% in CD133, and didn't further decrease at a concentration of 20 μ M.

In addition to the flow cytometric analysis, which assesses for CD133 on the cell surface, we evaluated the protein expression of CD133 in GSC11 and GSC23 cell lysates by western blot. Consistent with the flow cytometry analysis, the protein expression of CD133 was decreased in GSC11 and GSC23 following 72 hours treatment with C968 (10 μ M) (Figure 12C).

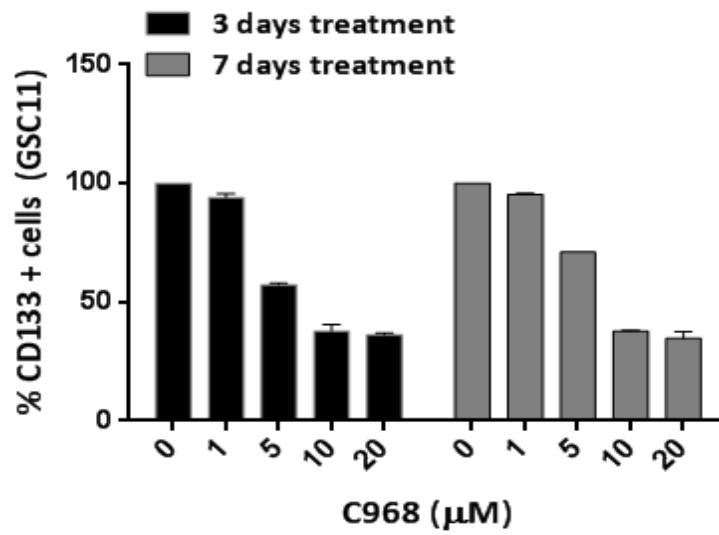
The suppression of CD133 suggested that glutaminolysis blockade could either have led to differentiation of the cells, or simply to the loss of stemness. In the case of differentiation, the CD133 expression would not reverse if the drug were removed, whereas the suppression wouldn't be drug dependent if there were a terminal differentiation of the GBM stem cells. Therefore, to determine if CD133 suppression was reversible, we treated GSC23 cells with C968 (10 μ M) for 7 days, then we washed the cells and cultured them without the drug. As previously shown, CD133 expression decreased from 70% to 21% with C968 (10 μ M), however, after 24 hours without C968, CD133 expression increased to 40% and further increased to 72% within 72 hours, which is comparable to the CD133 expression in the untreated cells (Figure 13). These data suggest that CD133 suppression is dependent on glutaminolysis blockade by C968 inhibitor.

Because, we had found that GSC23 and GSC11 cells proliferated slowly when treated with C968 (10 μ M), we investigated the impact of the CD133 reversibility on cells proliferation. We compared the cell number of GSC23 cells that were treated with DMSO, to GSC23 treated with C968 and GSC23 that were pretreated with C968 and then plated without C968. While GSC23 treated with C968 proliferated slowly in comparison to DMSO treated cells, GSC23 cells that were pretreated with C968 and plated without the inhibitor had an unaffected proliferation (Figure 14). Proliferation of the cells was reverted while the cells were cultured without C968, which also correlated with the reversibility of CD133 expression.

A



B



C

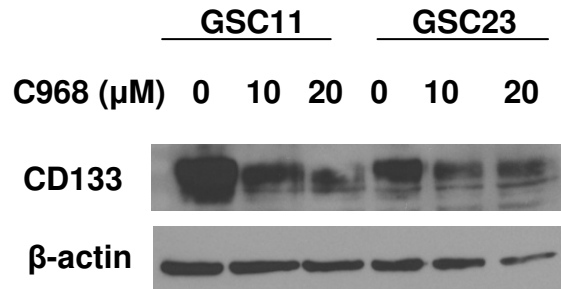


Figure 12. C968 suppresses the expression of CD133 stem cell marker. Neurospheres were dissociated with accutase and seeded at equal number, then treated with various concentrations of C968 (1-20 μM). Following 3 or 7 days of treatment, the cells were analyzed for CD133 expression by flow cytometry. (A) represents the expression of CD133 expression in treated GSC23, while (B) shows CD133 expression in GSC11. Bar graphs represent the mean \pm SD of three different samples.

(C) GSC11 and GSC23 cells were treated with C968 (10 and 20 μM) for 7 days. Cells were collected and total protein lysates were prepared. Lysates (20 μg) were subjected to western blotting to assess protein expression of CD133.

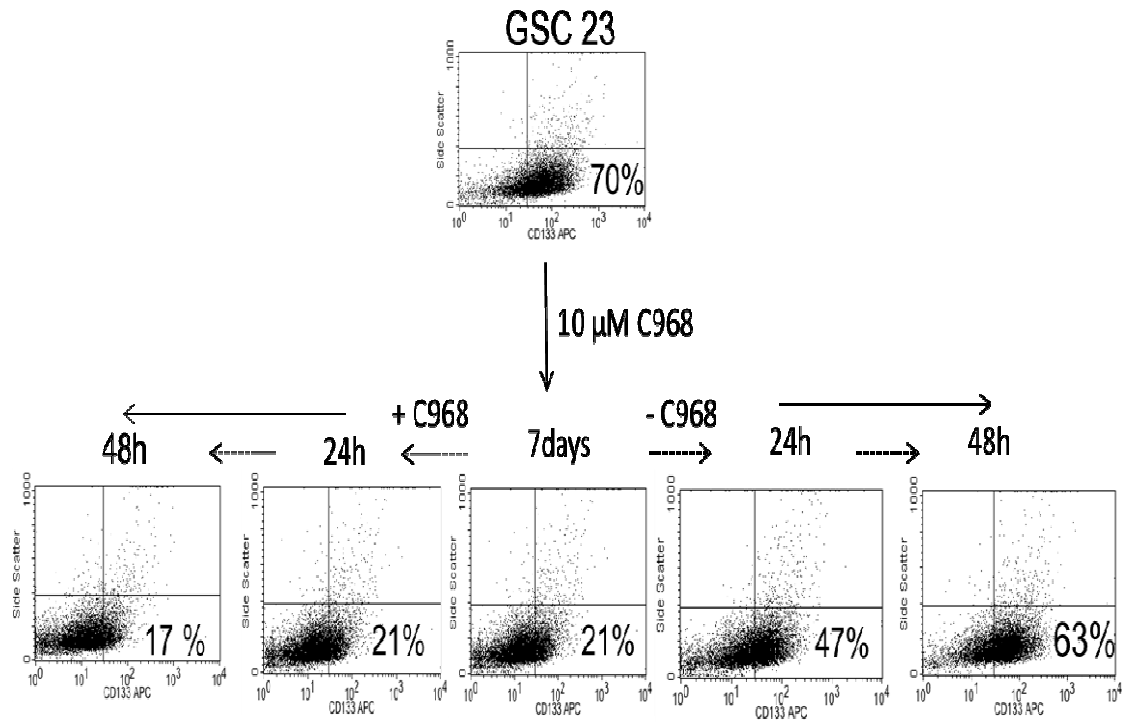


Figure 13. C968 reversibly inhibits CD133 expression. GSC23 cells were treated with 10 μ M of C968 for 7days, and then assessed for CD133 expression. The same cells were dissociated, washed with PBS and replated in fresh medium with or without C968. CD133 was assessed by flow cytometry after 24 hours and 48 hours. The dot plots represent APC-CD133 percentages as analyzed by flow cytometry.

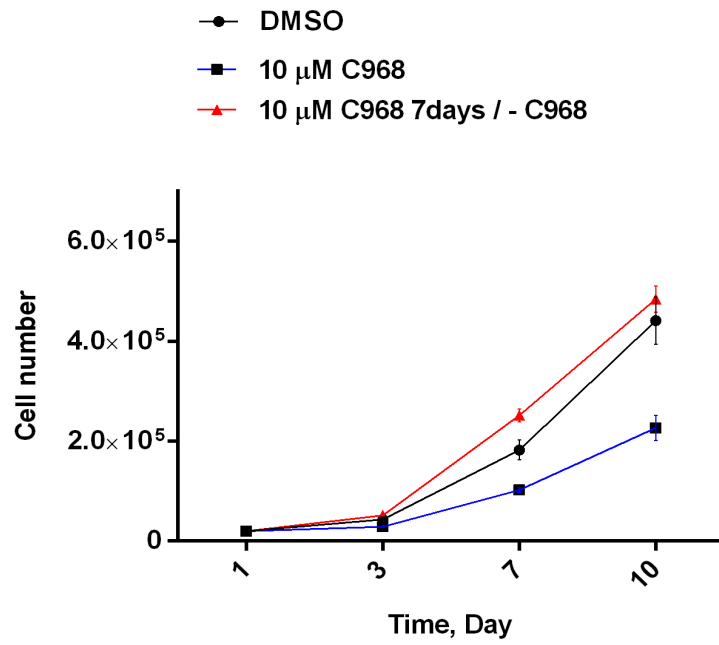


Figure 14. C968 withdrawal restores cells proliferation. GSC23 cells were dissociated with FBS and plated at an equal number in stem cells medium. Then the cells were either treated with DMSO (black) or 10 μ M of C968 (blue). GSC23 pretreated with C968 were also dissociated and plated at the same number without the drug (red). Cells were counted at the indicated times. Data points represent the mean \pm SD of biological triplicate.

Effect of C968 on the capacity for anchorage-independent growth

To assess the importance on glutaminolysis on the ability of GBM stem cells to grow in an anchorage-independent manner, GSC 23 and GSC11 cells were seeded in soft agar without or with C968 (10 μ M), after 4 weeks the colonies were stained with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide). Untreated GSC23 cells formed significantly more colonies, 400 colonies, compared to C968 treated cells which formed 50 colonies ($p=0.0006$). Similarly, GSC 11 formed about 650 colonies, whereas C968 treated cells formed less than 20 colonies ($p<0.0001$), as shown in (Figure 15).

Furthermore, to determine if the suppression of the colony formation was dependent on the continuous blockade of glutaminolysis by C968, we pretreated GSC23 cells with C968 (10 μ M) for 7 days, following which we plated the cells in soft agar in the absence or the presence of C968 (10 μ M). With C968 in the soft agar the ability of the cells to grown in an anchorage-independent manner was reduced. GSC23 cells formed less than 10 colonies, however, they formed an average of 650 colonies in the absence of C968 (Figure 16). These findings show that glutaminolysis is important for GBM stem cells to grow in anchorage-independent manner.

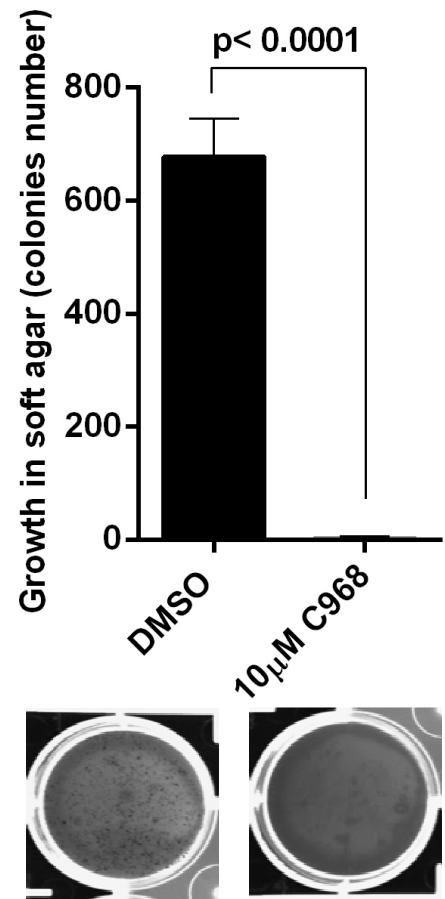
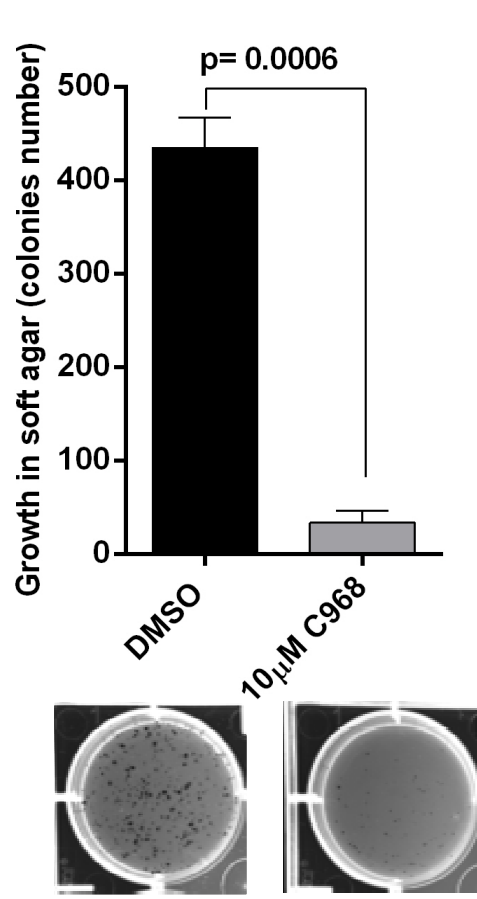


Figure 15. Decrease in cell colony formation with C968 treatment. A, GSC23 (10,000) were grown in soft agar (plus stem cells media) either in the absence or presence of 10 μ M of C968. Cells were maintained in culture for 4 weeks, after which they were stained with MTT (1 mg/ml) to visualize the colonies and colonies were counted (top). Results were calculated as means \pm SD of biological triplicate.

B, GSC 11 (10,000 cells) were grown in soft agar with or without 10 μ M of C968. After 4 weeks, cells were stained with MTT and colonies were counted. Results were calculated as means \pm SD of biological triplicate.

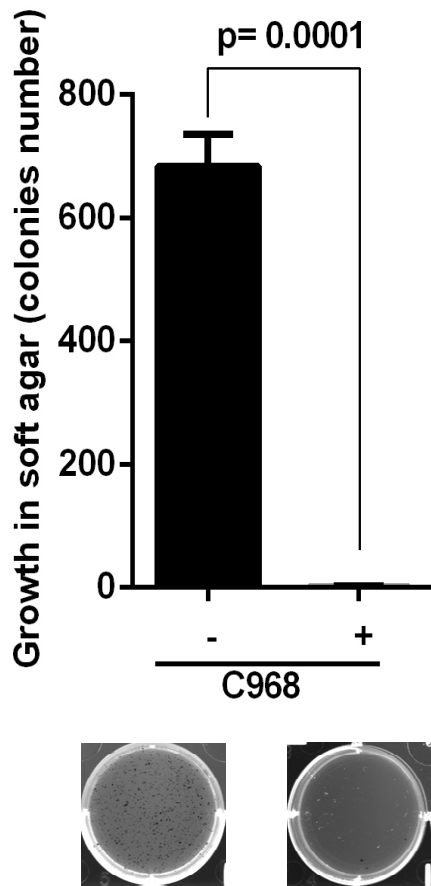


Figure 16. Anchorage-independent growth capacity is restored with C968 withdrawal.

GSC23 cells were treated with C968 (10 μ M) for 7 days, and then dissociated. 10,000 cells were seeded in soft agar (plus stem cells media) either in the absence or presence of 10 μ M of C968. Cells were maintained in culture for 4 weeks, after which they were stained with MTT (1mg/ml) to visualize the colonies (Bottom). Quantification of colonies is represented in the bar graph (Top). Results were calculated as means \pm SD of triplicate wells.

4.2 Discussion

Glutamine and glucose are two of the most abundant nutrients in the blood. Both are involved in cancer cells survival and proliferation in different types of cancer. However, little is known about the role they play in GBM stem cells. Our group has lead new research studies about the role of these nutrients in GBM stem cells. For instance, we showed that glucose affected GBM stem cells survival. Yuan and Wang showed that inhibiting glycolysis with 3-Bromo-2-oxipropionate-1-propyl ester (3-BrOP) in combination with a chemotherapeutic agent (BCNU) constituted a new strategy to eliminate GBM stem cells [95]. In this study we show for the first time that glutamine maintains proliferation and cell growth of GBM stem cells on soft agar.

Glutamine is an essential amino acid, cells uptake the glutamine needed to their function from the microenvironment [96]. Therefore, we measured the glutamine uptake in glioblastoma stem cells GSC23 and GSC11 and compared it to the cells that were induced to differentiate with FBS. We found that GBM stem cells consumed more glutamine than differentiated cells, which prompt us to determine the role of glutamine in GBM stem cells functions and maintenance.

In mitochondria glutamine is consumed by the cells and turned to glutamate by the glutaminase (GLS) enzyme, through a process called glutaminolysis. To obtain insight on how glutaminolysis potentially affects GBM stem cells, we employed a small inhibiting molecule previously identified as a GLS inhibitor, C968 [74].

As mentioned before GBM stem cells are characterized by their ability to form neurospheres, strikingly we observed that the treatment of GSC23 cells with C968 resulted in the formation of smaller neurospheres. To test whether the disturbance in neurospheres formation was a result of cell death or slower cell growth, we tested for cell viability and cell proliferation with glutaminase inhibitor, C968.

We found that when the cells were treated with 10 μ M C968, a concentration shown to inhibit GLS activity by Conroe et al., It didn't affect cells viability as shown by MTS assay and unlike in the glucose study which killed the cells (data not shown), glutaminolysis blockade didn't lead to cell death. Furthermore, we found that C968 treatment slowed from GSC23 and GSC11 cells proliferation. This demonstrates that glutamine doesn't affect GBM stem cells survival but affects cell proliferation.

Cells use glutamine as an energy source to promote proliferation. Glutamine is converted to glutamate through glutaminolysis, which is converted to α -ketoglutarate to produce ATP through the mitochondrial oxidative phosphorylation (Figure 1). Therefore, it was plausible to suggest that a depletion of ATP might be the cause of a slow proliferation. We tested whether C968 treatment depleted the cells from ATP, however, ATP generation wasn't compromised in the cells (appendix 1). Several studies showed that molecular pathway such as mTOR/AKT or TGF beta have been shown to affect stemness of GBM stem cells without killing the cells. The cells lose their stem cell markers and their ability to form tumors; therefore, we tested the effect of C968 on stemness.

CD133 (prominin1) stem cell marker is widely used to identify and isolate GBM stem cells; it is associated with stemness properties as self-renewal and increased tumorigenicity. The role of CD133 as a marker for cancer stem cells is still not fully understood and studies about its functions in GBM stem cells have been contradictory. In some cases CD133 negative population was able to form tumors as much as the CD133 negative population [97]. However, they are many studies that indicate that CD133 is important to the cells to maintain stemness and tumorigenicity. For instance, Paola Bresscia et al, [98] showed that suppressing CD133 expression using three different lentivirus-mediated short hairpin RNA, reduced GBM stem cells proliferation and tumorigenic capacity in xenograft mice.

In our study, we showed that inhibition of glutaminolysis by C968 suppressed the expression of CD133 stem cell marker at the plasmamembrane surface, as shown by flow

cytometric analysis, and in the cytosolic level as shown by western blotting for protein expression.

In addition, C968 is a reversible allosteric inhibitor of GLS, so it was important to test whether the suppression of CD133 was dependent on the C968 inhibitor and was temporary or if it made the cells definitely lose their stemness. Therefore, we treated the cells with C968 for 7 days before culturing them in fresh media with no drug. Following flow cytometry analysis, we determined that CD133 suppression was reversed when the drug inhibitor of GLS was removed from the cell culture.

Subsequently, we questioned if it had an effect on cell proliferation. Therefore, we treated the cells with C968 and then cultured them without or with C968. Our data showed that proliferation is no longer jeopardized when the cells are re-plated in a fresh medium. In fact, proliferation correlated with CD133 re-expression.

Importantly, blocking glutaminolysis prevented the cells from anchorage-independent growth, which was also reverted when the drug was removed from the soft agar. Taking together our data show that glutaminolysis might be regulating CD133 and mediating GBM stem cell growth.

It is worth to note that it has previously been shown that the transcription factor c-MYC induced glutaminolysis through the regulation of GLS expression via suppression of microRNA miR-23a/b, which normally suppresses GLS [76, 99]. Moreover, previous studies demonstrated that c-myc is critical to maintain GBM stem cells stemness and self-renewal without affecting cell survival [100]. Our finding that glutamine affects GBM stem cells stemness might support a mechanism by which c-MYC regulates GBM stem cells. This possibility will need to be further tested.

In this study we also explored some possibilities that lead to CD133 suppression. It was shown that an increase of reactive oxygen species (ROS) lead to loss of stemness and increase of differentiation in glioblastoma stem cells [101]. On the other hand, glutamine

catabolism to glutamate helps to support cells defense against oxidative stress through the formation of glutathione, which serves as a redox buffer [102], it was plausible that the inhibition of glutaminolysis with C968 caused an increase of ROS leading to a loss of stemness. However, our data showed that C968 didn't increase the mitochondrial ROS as measured by MitoSOX (appendix 2), therefore it couldn't support this possibility.

Moreover, CD133 suppression was reversible; we might speculate that it was epigenetically regulated. Previous studies have shown that CD133 can be regulated by epigenetic changes. For instance, the transforming growth factor beta (TGF- β) was shown to increase the expression of CD133 by increasing demethylation [103]. On the other hand, a previous report showed that C968 treatment in breast cancer cell lines could induce methylation [104], therefore it was plausible that CD133 suppression was caused by methylation. To test this possibility we co-treated GSC23 with C968 and an inhibitor of DNA methylation, 5-aza-2'-deoxycytidine [105]. Flow cytometry analysis for CD133 showed that 5-aza-2'-deoxycytidine didn't prevent CD133 suppression by C968, which suggests that methylation wasn't the cause of CD133 suppression. This will need further studies with different approaches such as epigenetic sequencing, because CD133 has 3 promoters and it might be complicated to prevent its methylation with one agent [106].

Furthermore, CD133 is a plasmamembrane protein which is translated in the cytosol and sent to the cell surface. Glutamine, besides glucose and Acetyl Coenzyme A (Ac-CoA) are required for N-linked glycolysation, folding and trafficking of growth factor receptor to the cell surface. It is plausible that blocking GLS affects N-linked glycolysation. This possibility remains to be tested in the future.

In Conclusion, our study has shown that inhibiting glutaminolysis by C968 slowed GBM stem cells growth and prevented GBM stem cells from forming colonies on soft agar. In addition, proliferation and anchorage-independent capacity of GBM stem cells correlated with CD133 expression, supporting the importance of CD133 as a stem marker in GBM stem cells.

This study shows for the first time that glutamine metabolism affects GBM stem cells and offer opportunities to further investigate the molecular bases underlining the regulation of CD133 by glutaminolysis. It will also be important to determine if the loss of CD133 caused by blocking glutaminolysis with C968 could sensitize GBM stem cells to conventional chemotherapies.

CHAPTER 5. Mevalonate pathway as a therapeutic target

5.1 Results

Identification of mevalonate pathway as a target for GBM stem cells

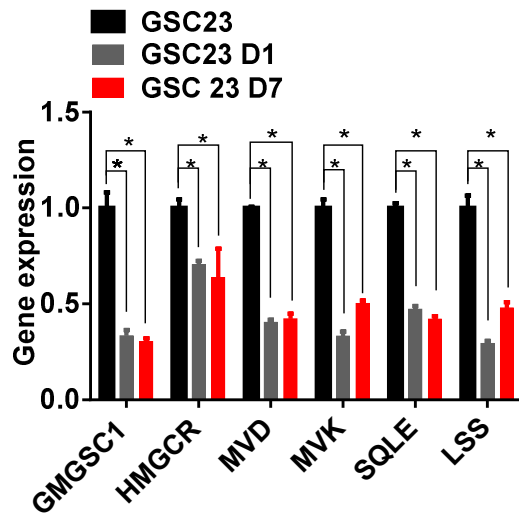
We sought to unravel key genes and molecular pathways important to glioblastoma stem cells using high-throughput microarray technology. We compared the gene expression profile of GSC11 cells to FBS induced cells at 72 hours. We identified the mevalonate pathway as being differentially modulated between the stem cells and differentiated counterparts. For instance, the expression of mevalonate pathway related enzymes correlated inversely with differentiation, such that genes associated with this pathway were elevated in the GSC cells compared to their differentiated counterpart (FBS induced cells) as early as 24 hours (Table 3).

To further validate these results quantitatively, we analyzed *HMGSC1*, *HMGCR*, *MVD*, *MVK*, *SQLE*, *LSS*; transcripts levels, by quantitative real-time polymerase chain reaction (QRT-PCR), in GSC23 and GSC11 glioblastoma stem cells cultured in serum free medium or in medium containing 5% FBS for 24 and 72 hours, noted respectively as D1 and D3. Exposure to FBS significantly decreased the mRNA expression levels of these genes in both glioblastoma stem cell lines as early as 24 hours post-exposure to FBS ($P < 0.005$) (Figure 17).

Table 3. Mevalonate pathway related genes were significantly downregulated after differentiation with FBS.

Gene	Fold change (day3 vs Control)
GFAP	2.384728
PROM1	-1.92046
MVK	-1.6279
DHCR24	-1.77728
SOX2	-1.7935
MVD	-2.05976
DHCR7	-2.31148
LSS	-2.72754
HMGCR	-3.01903
FASN	-3.10088
SQLE	-4.33563
FDFT1	-5.3944
OLIG2	-8.47662
HMGCS1	-9.02319
INSIG1	-9.02413
ALDOC	-9.24284

A



B

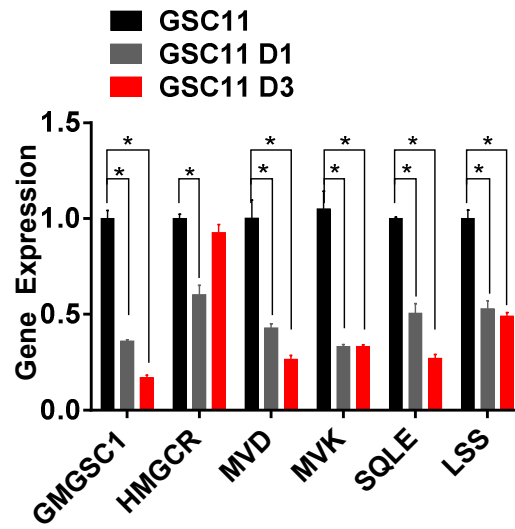


Figure 17. Mevalonate pathway is upregulated in glioblastoma stem cells. (A) GSC23 and FBS induced cells for 24 hours and 7 days, (B) GSC11 and FBS induced for 24 and 72 hours were collected and total RNA was extracted, revers-transcribed, and analyzed by real time PCR for transcripts level for mevalonate pathway associated genes. All expression levels were normalized internally to β -actin and FBS induced samples were normalized to the no FBS samples. The error bars represents the standard deviation (* $p > 0.005$).

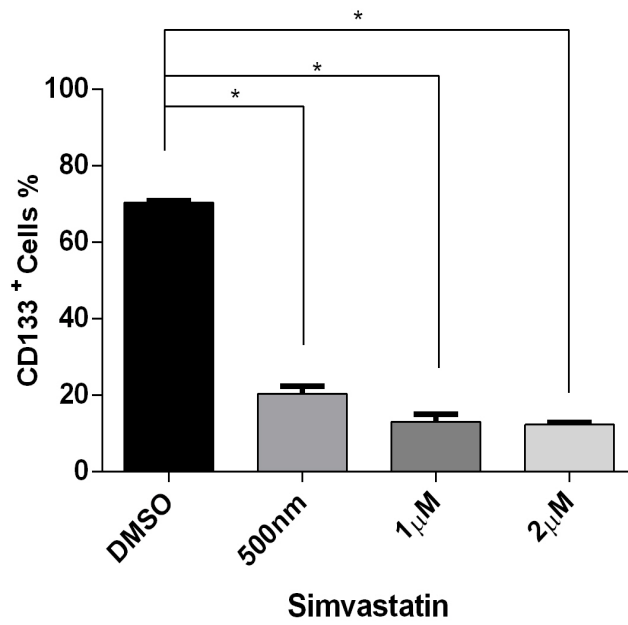
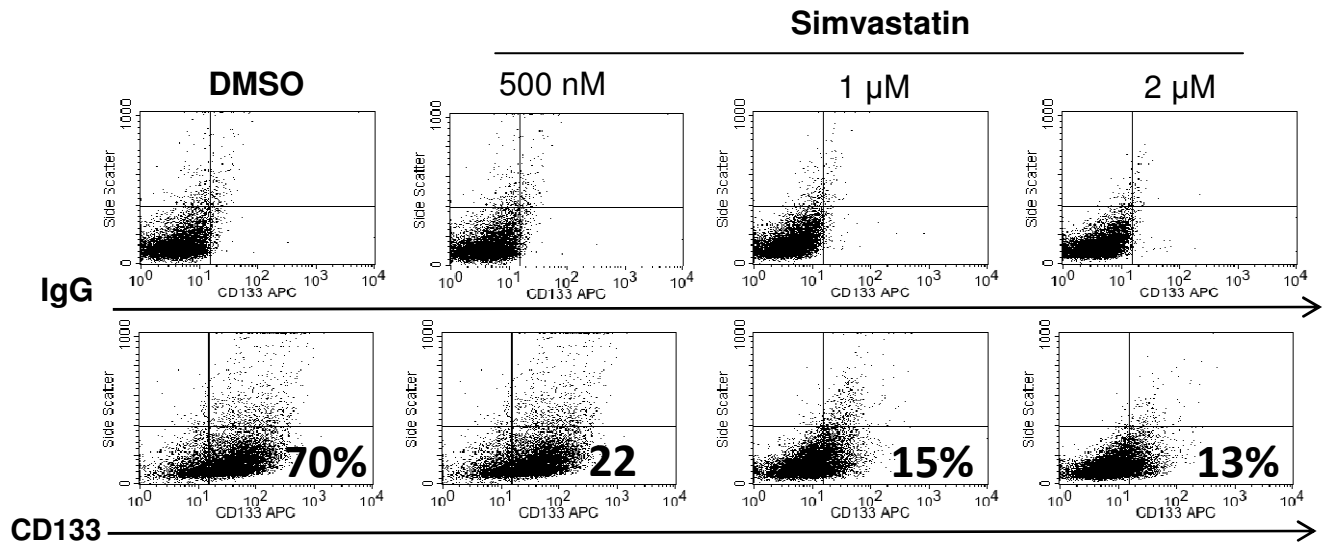
Blocking mevalonate pathway caused cell death in GBM stem cells

The reduction in mevalonate related genes expression with differentiation suggests that mevalonate pathway preferentially associated with the stem cell subsets. Therefore, we hypothesized that mevalonate might regulate the stemness or the survival of GBM stem cells.

To investigate the role of mevalonate pathway in GBM stem cells, we tested the effect of blocking this pathway with simvastatin on CD133 positive cells. As mentioned before, simvastatin is widely used as cholesterol lowering drug which works through inhibiting HMG-CoA reductase activity. GSC23 and GSC11 cells were treated with 500 nM, 1 μ M or 2 μ M simvastatin for 3 or 5 days, respectively. Cells were labeled with CD133-APC and analyzed by flow cytometry. We observed a dose dependent decrease of the CD133 positive population. 500 nM simvastatin decreased CD133 by 75% in GSC23 and 80% with a concentration of 1 and 2 μ M after 3 days treatment. Whereas, after 5 days treatment of GSC11, 500 nM simvastatin decreased 50% of the CD133 positive population, and 63% and 82% with 1 μ M and 2 μ M of simvastatin, respectively (Figure 18 A-B).

These observations suggested that simvastatin might exert a cytotoxic effect on GSC23 and GSC11 cells. We further assessed for cell viability by measuring the mitochondrial membrane potential using the fluorescent dye Rhodamine 123, because the loss of mitochondrial potential is a mark of cell death. We treated GSC23 cells with simvastatin 500 nM, 1 μ M or 2 μ M for 3 or 5 days. We observed about 40% of cell death with 500nM of simvastatin after 3 days and about 90% after 5days. 1 μ M simvastatin caused 50% of cell death after 3 days, and about 95% after 7days (Figure 19).

A



B

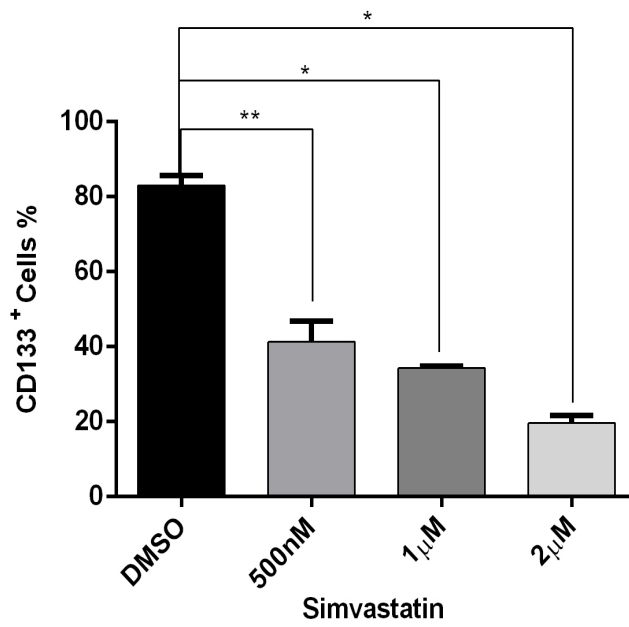
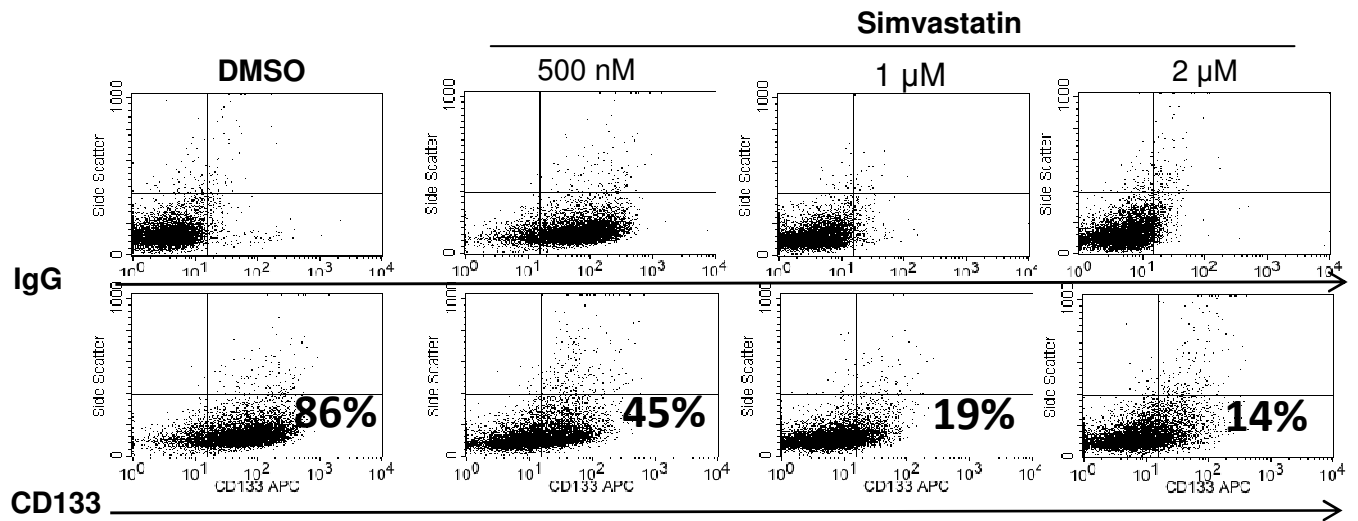


Figure 18. Simvastatin depletes CD133 positive population. (A) GSC 23 were dissociated to single cells, an equal number of cells were plated and treated with DMSO or 500nM, 1 μ M or 2 μ M of simvastatin. After 5 days the cells were analyzed for CD133 expression by flow cytometry. Top plots are representative data from the CD133 analysis. Values depict the percentage of CD133 positive population. Bottom graphs represent the mean of biological triplicate \pm SD.

(B) GSC11 cells were treated with DMSO, 500nM, 1 μ M or 2 μ M of simvastatin. After 7 days CD133 expression was determined by flow cytometry analysis. Top plots are representative of the flow cytometry analysis. Values depict the percentage of CD133 positive population. Bottom graphs represent the mean of biological triplicate \pm SD.

(A,B) * $p < 0.0001$, ** $p = 0.003$

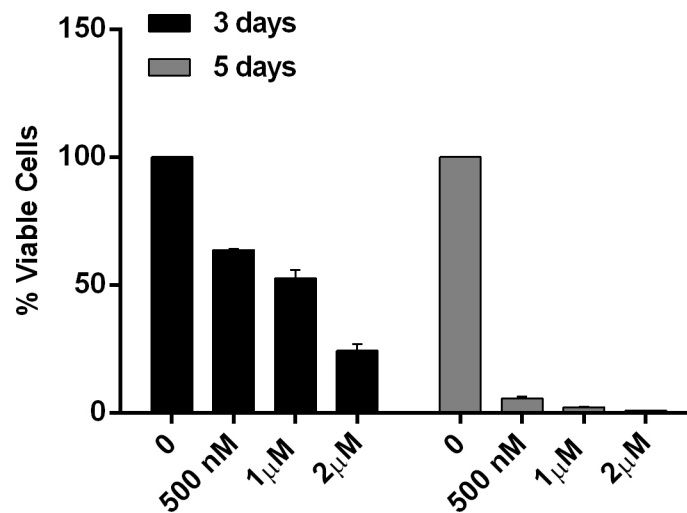


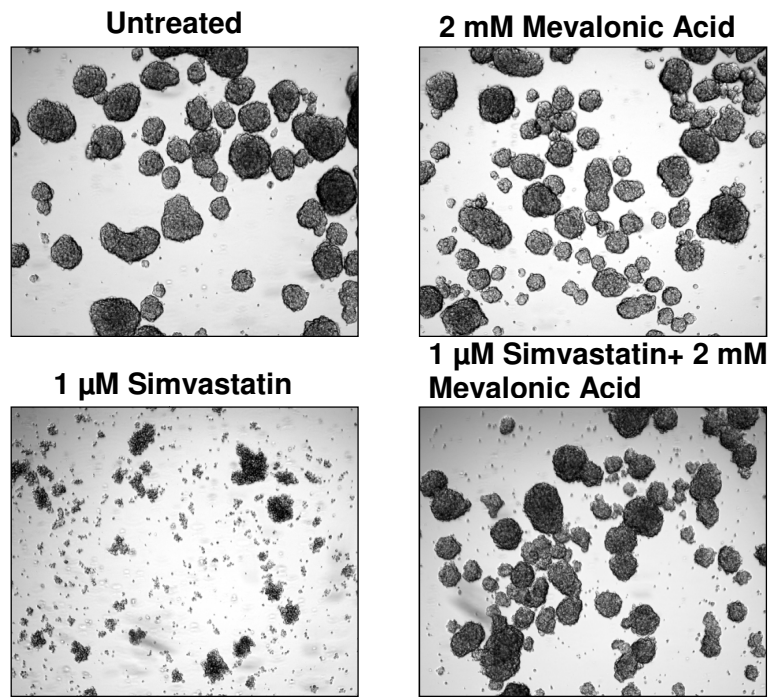
Figure 19. Simvastatin induces cell death in glioblastoma stem cells. GSC23 were dissociated and same number of cells was plated. The cells were treated with DMSO (0), 500 nM, 1 or 2 μ M of simvastatin for 3 or 5 days. Mitochondrial membrane potential was assessed by incubation of the cells with Rhodamine 123 for one hour followed by flow cytometry analysis.

Mevalonic acid is able to prevent cell death by simvastatin, demonstrating that simvastatin is specifically targeting the mevalonate pathway.

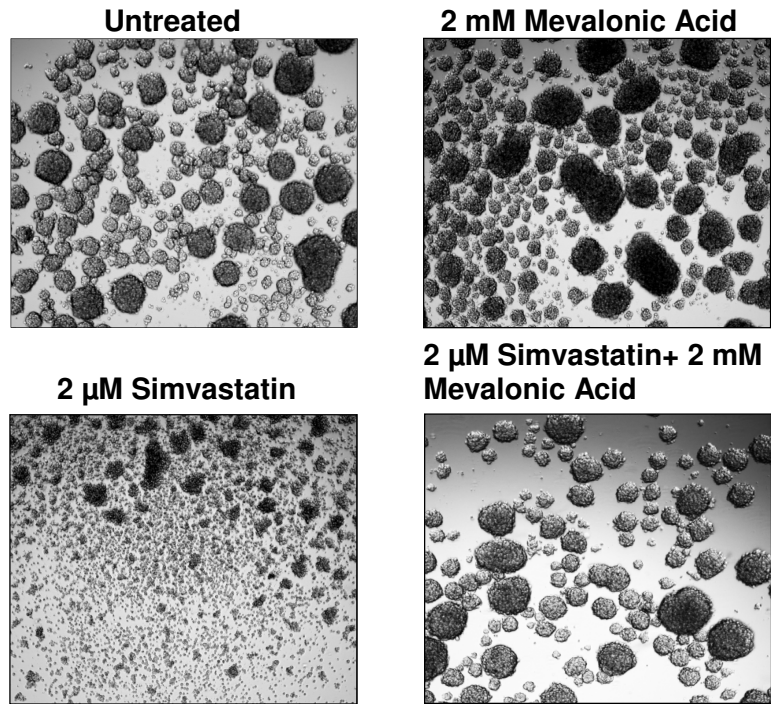
To insure that simvastatin was specifically blocking the mevalonate pathway, by inhibiting the HMG-COA reductase, the cells were treated with either 2 mM of mevalonic acid, 1 μ M of simvastatin or a combination of mevalonic acid and 1 μ M Simvastatin. While we observed a loss of neurospheres in simvastatin treated cells, the co-treated with mevalonic acid kept intact neurospheres (Figure 20 A-B).

We further assessed for cell death by measuring mitochondrial membrane potential in this above experimental setting. Indeed, mevalonic acid wasn't toxic to the cells, and the cells treated with simvastatin lost all potential, whereas the cells co-treated with mevalonic acid preserved their membrane potential. In other words, mevalonic acid prevented simvastatin to cause cell death (Figure 20C)

A



B



c

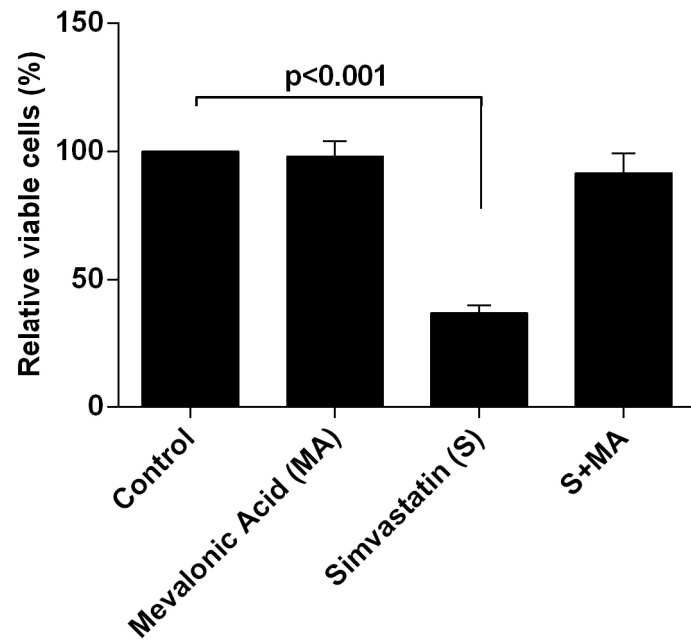
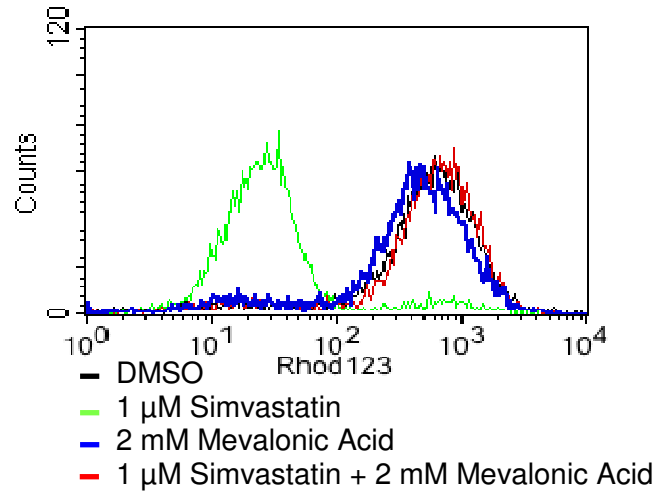


Figure 20. Mevalonic acid prevents simvastatin induced cell death. Cells were dissociated with accutase and plated at an equal number. Cells were treated for 5 days then observed under microscope.

(A) GSC23 cells were treated with 2 mM mevalonic acid, 1 μ M simvastatin, or a combination of mevalonic acid and simvastatin.

(B) GSC11 cells were treated with 2 mM mevalonic acid, 2 μ M simvastatin, or co-treated with mevalonic acid and simvastatin.

(C) GSC23 cells were treated with 2 mM mevalonic acid alone or 1 μ M simvastatin or with both simvastatin and mevalonic acid. To assess for cell death, cells were analyzed for loss of mitochondrial membrane potential. Top histogram is representative of the Rhodamine 123 analysis by flow cytometry. Bottom quantitation graphs represent means of biological triplicate samples \pm SD.

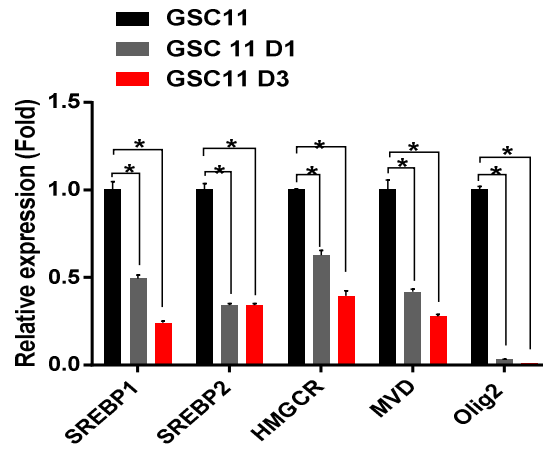
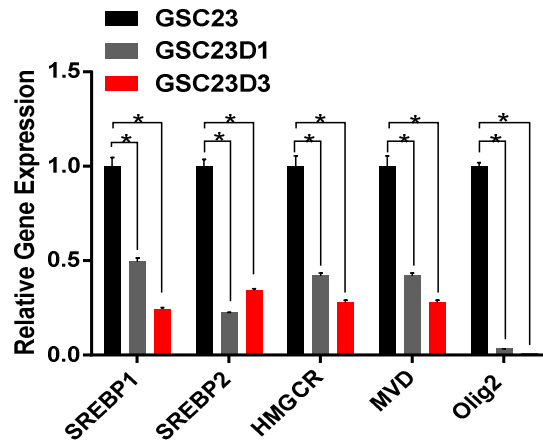
Mevalonate transcription is tightly regulated by SREBP

Sterol regulatory element-binding proteins 1 and 2 (SREBP 1 and SREBP2) are transactivation factors that modulate and activate the transcription of mevalonate genes. To determine if SREBPs were modulated, we analyzed SREBP1 and SREBP2 transcripts levels, by QRT-PCR in GSC11 and GSC23 cells in comparison to the FBS induced cells. Interestingly, SREBP1 and SREBP2 expressions were elevated in GBM stem cells as compared to FBS induced cells (Figure 21 A).

Inhibiting SREBP using a small molecule Betulin also caused cells death

Because SREBP high expression was associated with mevalonate expression pathway in GBM stem cells, we sought to block the mevalonate pathway by inhibiting SREBP. Therefore, the cells were treated with betulin, a small molecule inhibitor of SREBP (Figure 21B). Betulin was previously identified by Jing-Jie Tang et al., from a mass screening as a specific inhibitor of SREBPs [107]. GSC23 cells were treated with 3 and 6 $\mu\text{g/ml}$ of betulin for 7 days. Subsequently, we observed that GSC23 neurospheres were lost (Figure 22). We furthermore assessed for cell viability by measuring the mitochondrial membrane potential with Rhodamine 123. We observed a concentration dependent loss of cell viability with Betulin treatment, with a total loss at 6 $\mu\text{g/ml}$. Note that there was no cell death at earlier time (data now shown).

A



B

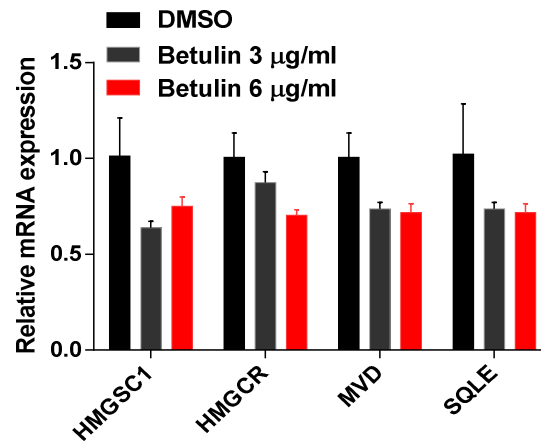
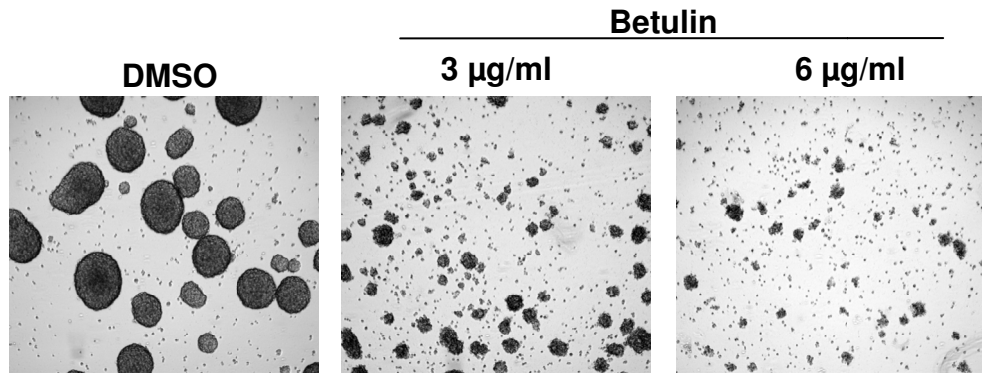


Figure 21. SREBP mRNA levels correlate with mevalonate genes expression and their decrease with Betulin treatment. A, SREBP transcripts levels were compared by real time PCR in (Top) GSC 23 (Bottom) GSC11 that were cultured as stem cells or induced with FBS *p< 0.0001.

B, GSC23 were treated with Betulin 3 and 6 µg/ml for 7 days. RNA was extracted, revers transcribed and transcripts levels for mevalonate pathway were assessed by QRTPCR

A



B

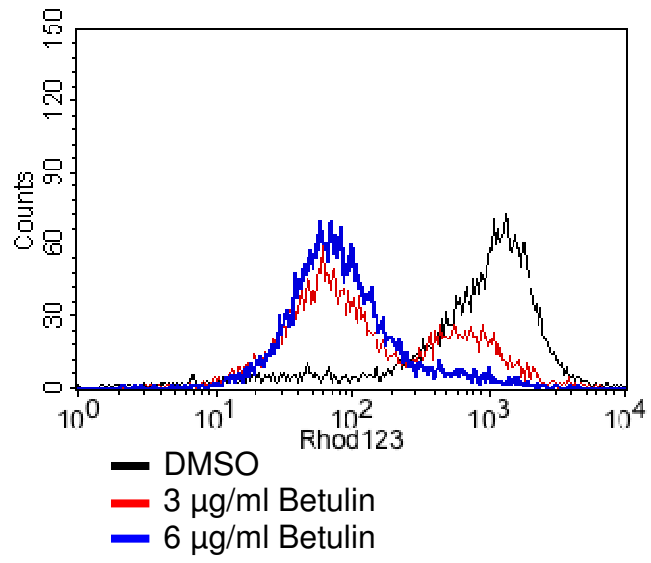


Figure 22. Betulin treatment induces GBM stem cells death. GSC23 were plated and treated with 3 or 6 $\mu\text{g/ml}$ of betulin for 7 days. A, cells were observed under the microscope. B, GSC23 were treated with betulin for 7 days, then assessed for mitochondrial membrane potential with Rhodamine 123. Dead cells lose their membrane potential which is represented by a shift toward the left of the control DMSO treated cells.

5.2 Discussion

Glioblastoma is the most lethal form of brain tumors with a median survival of 12 months. Patients with glioblastoma not only don't respond to therapies, but they also experience relapse. A line of studies have suggested that glioblastoma stem cells contribute to the pathogenesis of the disease, may mediate tumor recurrence, and are resistant to conventional therapies. Therefore, we are urged to identify pathways that are important for GBM stem cells survival to serve as new therapeutic targets.

In this study we aimed to identify a new metabolic pathway to be targeted in GBM Stem cells. Toward this goal, we analyzed gene expression modulation in GBM stem cells (GSC11) and FBS induced cells for 24 and 72 hours. The analysis revealed that the expression of genes related to the mevalonate pathway were elevated compared to the FBS pathway), which were further validated by real time PCR for HMGSC1, HMGCR, MVD, MVK, SQL, LLS.

Mevalonate pathway was shown to be hyper-activated in some cancer and is implicated in tumor progression by contributing in cancer cells proliferation, invasiveness and survival [79]. Statin drugs are known to inhibit the rate limiting enzyme, HMG-CoA, in the mevalonate pathway leading to decrease of the cholesterol biosynthesis. Therefore, we utilized the statin drug simvastatin to explore the effect on GBM Stem cells [78].

We found that there was a depletion of CD133 positive cells with simvastatin. GSC23 and GSC11 treated with simvastatin had a decreased CD133 expression starting from a concentration of 500 nM, which further decreased with 1 μ M of simvastatin. These results hinted us toward two possibilities: 1) that simvastatin leads to the loss of stemness, as seen with C968 or 2) Simvastatin caused cell death.

We found that simvastatin caused cell death in GBM stem cells as shown by the loss of mitochondrial membrane potential. Consistently with previous studies [89, 108], It is important

to note that GSC23 cells were resistant to temozolomide *in vitro*, a conventional chemotherapy for the treatment of glioblastoma (Appendix 3),

Contrary to other studies in breast cancer cell lines and glioblastoma cell lines in which 10 μM of simvastatin was needed to induce cytotoxicity, we observed cell death in GBM stem cells starting from 500 nM. Moreover, FBS induced cells were also treated with Simvastatin, the cells were induced for 7 days with FBS then treated with either 1 μM for a week, following which the cells were analyzed for cell death using Rhodamine 123. We didn't observe cells death at this concentration. This suggests that mevalonate pathway is perhaps even more active in GBM stem cells rendering them more sensitive to simvastatin. Importantly we demonstrated simvastatin specificity to inhibit mevalonate in GBM stem cells. We co-treated the cells with mevalonic acid was able to prevent cells death, demonstrating that simvastatin was specifically inhibiting the mevalonate pathway.

Previous studies showed that statin drugs induction of cell death in breast cancer and glioblastoma was independent from its property to lower cholesterol synthesise and was directly linked to intermediates linked to protein isoprenylation such as geranygeranyl phosphate and farnesyl phosphate [84]. However, in our study co-treatment of the GSC23 cells with geranygeranyl pyrophosphate or farnesyl pyrophosphate failed to prevent death induced by simvastatin (Appendix 4). Furthermore, the main pathway being the synthesis of cholesterol, we co-treated the cells with a direct precursor of cholesterol biosynthesis, squalen, unfortunately squalen was toxic to the cells, which is probably due to the high volume needed to add to the cells culture to reach the appropriate concentration. To determine if cell death is induced by the decreasing levels of cholesterol remains to be investigated using different approaches.

Because it was also reported that simvastatin treatment increased ROS production in breast cancer cells, leading to cell death, we measured ROS in GSC23 treated with simvastatin [84]. However, we didn't observe an ROS increase.

Furthermore, mevalonate enzymes expression is regulated by transcription factors, SREBPs. A study showed that mevalonate pathway was important to protect mammary tissue architecture, by upregulating SREBPs expression [87].

Interestingly we found that the transcription factor SREBPs transcripts were higher in GBM stem cells, and associated with a high mevalonate pathway. SREBPs mRNA expression levels were elevated in GBM stem cells, and downregulated with FBS. Therefore, we targeted the mevalonate pathway upstream by inhibiting SREBPs, using betulin inhibitor. Similarly to simvastatin, we found that betulin killed GBM stem cells at concentrations previously reported to downregulate SREBP [107]. In conclusion, in this study we identified the mevalonate pathway as a target to eliminate glioblastoma stem cells.

CHAPTER 6. Research summary

6-1 Experimental summary

Glioblastoma remains the most lethal form of brain tumors; moreover, GBM stem cells have been implicated in tumor initiation, resistance to chemotherapies leading to recurrence of the tumor. Therefore, it is crucial to understand the biology of these cells. In this study, we investigated the metabolic profile of cancer stem cells, and the importance of two metabolic pathways namely, glutamine and mevalonate pathways in maintaining GBM stem cell growth, expression of stemness, and viability.

First, based on previous knowledge that GBM stem cells differentiated when exposed to fetal bovine serum, we could compare the mitochondrial respiration in two cell lines in their stem cell state and in their differentiated phenotypes. We found that during differentiation GBM stem cells shifted toward a higher oxygen consumption, which didn't result from an increase of mitochondria biogenesis, but probably from an increase of the TCA cycle substrate, α -ketoglutarate, which could be produced from an increase of the glutaminolysis, since we found that GLS2 enzyme expression was unregulated. This finding support previous reports suggesting that stem cells might be present in hypoxic niches, whereas differentiated cells are exposed to more O_2 . Furthermore, cancer cells are characterized by metabolic reprogramming, unlike normal cells, cancer cells use aerobic glycolysis to generate energy [109]. In our study we showed that cancer stem and differentiated cells are also different in their bioenergetics profile, which could help us develop new therapeutic strategies.

We also found that glutaminolysis was important to maintain cell growth, stemness and clonogenicity of GBM stem cells. Treatment with glutaminase inhibitor C968, lead to a suppression of the stem cell marker CD133, which was accompanied by a decrease in the proliferation and the capacity for independent-anchorage growth.

Lastly, we unraveled a metabolic pathway that is highly associated with GBM stem cells. The mevalonate pathway was upregulated in both GSC cell lines. We further determined the biological relevance of this pathway in GBM stem cells by using a statin drug. We showed that a commonly used drug to lower cholesterol, simvastatin, was able to cause cell death in GBM stem cells. It was previously shown that simvastatin can cause cell death in breast cancer and breast cancer stem cell by inhibiting the generation of isoprenoids, however, in GBM stem cells, simvastatin induced cell death was independent from isoprenoids biosynthesis.

This study brings new insights into roles that metabolism could play in GBM Stem cells, and provide a good support for the development of new studies to further determine how metabolic shift contributes to differentiation or to underline the mechanism by which glutamine metabolism regulates CD133.

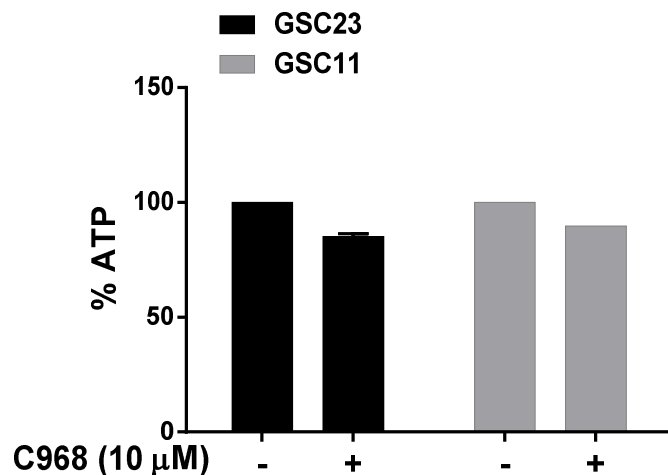
6-2 Future directions

Results from this study showed a mitochondrial shift during differentiation, however, we don't know if it was a primary event in the differentiation. Also, we don't know if the increase of mitochondrial respiration is necessary for GBM stem cells differentiation. Since we showed an increase of GLS2 protein expression during differentiation, we would test if overexpressing this gene in GSCs, would lead to differentiation. Moreover, we would test if blocking mitochondrial respiration would prevent differentiation.

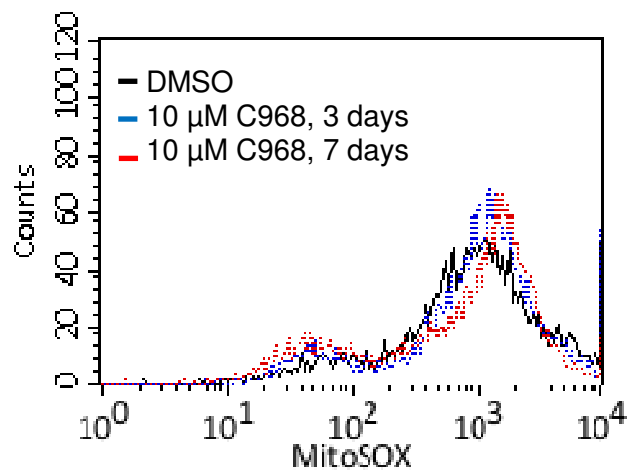
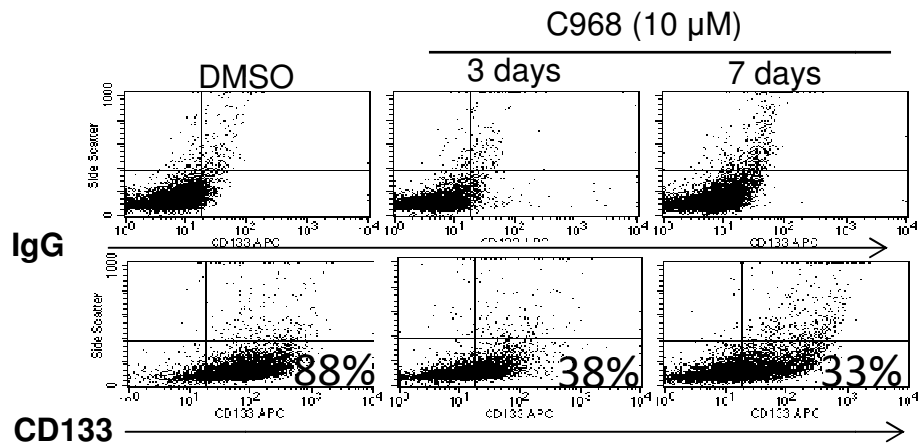
Another part of our study was to investigate the role of glutamine in GBM stem cells. We showed that C968 inhibited CD133, however the mechanism remains unknown. We would test the effect of C968 on methylation of CD133, because it was shown that hyper-methylation suppresses CD133 expression. Furthermore, blocking glutaminolysis with C968, slowed proliferation, and significantly lowered clonogenicity, but did not lead to cell death. It is important to test if the suppression of CD133 in GSCs, would sensitize the cells to other chemotherapies. We would combine C968 to other drugs, such as temozolomide, and test for cell death.

Furthermore, we showed that simvastatin caused cell death *in vitro*, it would be important to study its effectiveness *in vivo*. We would use glioblastoma xenografts to treat with simvastatin. In addition, we don't know if other statin drugs would be effective to eliminate GBM stem cells, or if it is specific to simvastatin. Therefore, we would test lovastatin in future experiments.

CHAPTER 7. Appendix

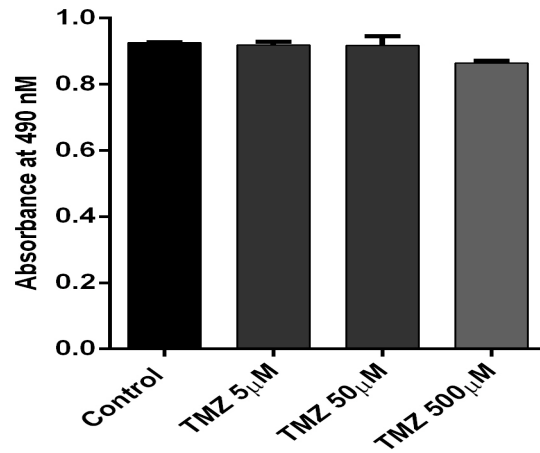


Appendix 1. C968 doesn't affect ATP production. GSC23 (black) and GSC11 (grey) cells were dissociated and plated at a density of 25,000 cells per well in a 96 well-plate. Cells were left untreated or treated with 10 μM C978, and incubated for 6 hours following which ATP was measured. Bar graphs represent the mean \pm DS of biological triplicate.

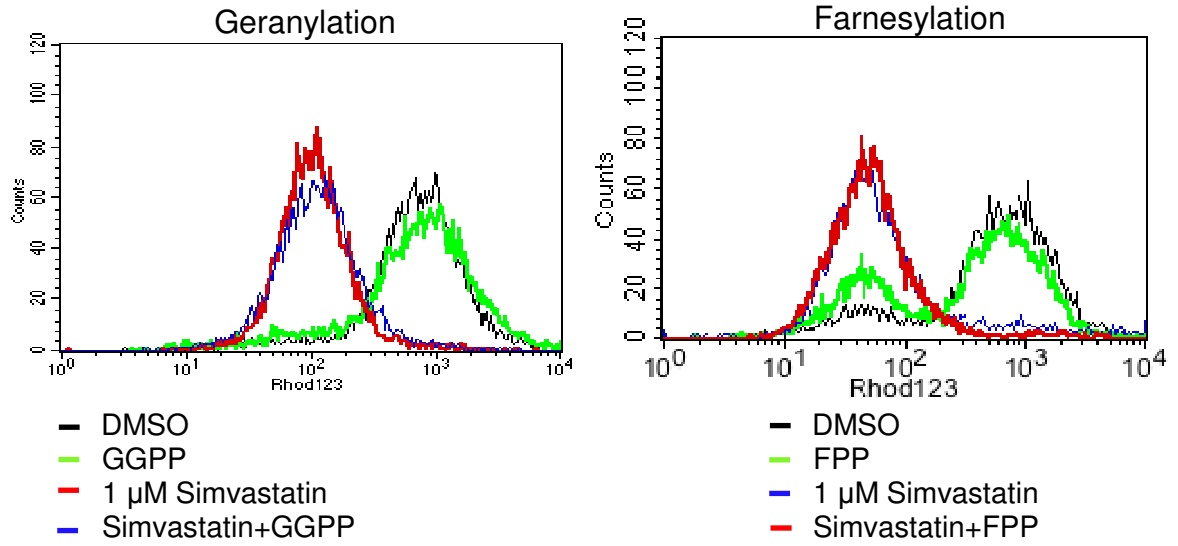


Appendix 2. C968 doesn't increase from ROS production in the cells. Dot plots (top) represent CD133 expression in GSC23 with DMSO or with C968 (10 μM) treatment for 3 or 7 days.

Histogram (bottom) represents mitochondrial ROS in the same cells. Cells were treated with C968 for 3 or 7 days, after which ROS was measured by flow cytometric analysis with MitoSox dye as compared to DMSO treated cells (black).



Appendix 3. GSC23 cells are resistant to temozolomide. GSC23 were seeded in a 96 well-plate at a density of 5,000 cells per well and then treated with various concentrations of temozolomide (TMZ). 40 μ l MTS was added in each well and the plates were incubated for 3 hours. Absorbance was measured at 490nm. Bars represent the mean of triplicate wells.



Appendix 4. GGPP and FPP don't prevent cell death induced by simvastatin in GSC23.

GSC 23 cells were treated with combinations of simvastatin, GGPP and Simvastatin with FPP.

Mitochondrial membrane potential was assessed to determine the viability of the cells.

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Vita

Naima Hammoudi was born in Algiers, Algeria on July 09, 1984, the daughter of Chafika Kessi Hammoudi and Boualem Hammoudi. She completed her education at Université des Sciences et de la Technologie Houari Boumedienne, Algiers, Algeria. In 2005, she received her Bachelor of Science with a major in Microbiology. She spent the following year volunteering at Pierre et Marie Curie hospital in Algiers, where her desire to pursue cancer research matured. She moved to the U.S.A in 2006, where she conducted research work before she joined The University of Texas Health Science Center in Houston in 2007.