Role of ER-Mitochondria interface in the regulation of Glioma Stem Cells

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Research Article

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Abstract

Purpose: Glioblastoma (GBM) treatment is extremely challenging due to the high complexity of the tumor, being one of the tumors in which a subpopulation of highly resistant cancer initiating cells (GICs) has been clearly identified. Thus, understanding the differences between GICs and tumor bulk cells is therefore essential to move to less conventional but more efficient approaches.

Methods: Fluorimetry was used to measure glucose uptake, mitochondrial calcium, intracellular oxidants and mitochondrial membrane potential. Spectrophotometry was used to monitor lactate dehydrogenase activity. Self-renewal was determined by the limiting dilution assay and cell death by trypan blue exclusion assays. Protein expression was determined by western blot while gene expression was determined by real-time PCR.

Results: We found that, unlike their differentiated progeny, GICs survival and stemness depend on mitochondrial metabolism. GICs present higher glucose uptake and mitochondrial membrane potential and less LDH activity, being more sensitive to mitochondrial inhibition than their differentiated counterparts. Calcium flux seems to play an essential role in the maintenance of this distinct metabolic phenotype with a decrease in the expression of VDAC and Grp75, two of the main proteins in the transfer of calcium from endoplasmic reticulum (ER) to the mitochondria. Disruption of ER homeostasis using ER stress inducers or inhibition of ER-mitochondrial contact sites using the Grp75 inhibitor MKT-707 resulted in GICs cytotoxicity and loss of stemness. Moreover, MKT-077 also potentiates the effect of temozolomide, current treatment for glioblastoma.

Conclusions: Our data indicates that ER-mitochondrial homeostasis is essential for regulation of GICs metabolism, survival and stemness.

1. Introduction

Glioblastoma (GBM), the most frequent and most lethal primary malignant brain tumor, represent a highly complex tumor consisting in cancer cells and various non-neoplastic cells [1, 2]. This complexity represents a challenge to achieve an effective therapy to overcome the current median survival of 14 months even after conventional therapy which combine surgical resection, radiotherapy and chemotherapy [3]. Additionally, GBM is one of the tumors in which a subpopulation of cancer initiating cells with stem cells properties has been clearly identified. This glioma initiating cells (GICs) and their differentiated counterpart (tumor bulk cells) may represent opposite extremes of cells forming the highly heterogenous GBM mass in vivo [4, 5]. GICs not only resist current treatments and repopulate the tumor but are also able to evade the host immune system. Thus, the greater proportion of GICs, the higher tumor aggressivity and poorer prognosis [6], as current therapies show poor efficacy against GICs [4, 6]. For these reasons, understanding the differences between both types of cancer cells and the heterogenicity of the tumor could help to move cancer therapy towards less conventional but more efficient approaches.
It must also be considered that the subpopulation of cancer stem cells is not “immovable” and that new stem cells may appear depending on multiple factors. Evidence suggests that stem cell properties can be acquired as a consequence of mutations and metabolic changes occurring in normal stem cells or differentiated cancer cells that move up the cancer cell hierarchy for their expression of pluripotent genes. In this sense, many of the identified cancer stem cells biomarkers has some role in cellular metabolism. These metabolic changes, capable of inducing CSCs reprogramming are collectively called "metabostemness" [7].

Loss of control of mitochondrial metabolism is one of the main hallmarks of cancer [8]. Many tumor cells, including GBM, have an altered glucose metabolism known as Warburg effect [9], which favors the transformation of pyruvate to lactate instead of its incorporation into the tricarboxylic acid cycle and the subsequent electron transport chain in the mitochondria. This metabolic adaptation, although it is energetically less favorable, has other advantages for tumor cells in terms of redox balance and intermediate metabolites synthesis [10]. However, while Warburg effect has been described for GBM, data seem to indicate that metabolism could vary between different tumor populations and can be also modulated within same tumor population by tumor microenvironment [11]. Thus, a recent report showed that GICs are less glycolytic than differentiated glioma cells [12]. But on the other hand, there is also abundant literature that supports aerobic glycolysis as the main bioenergetic source in CSCs of various tumor types including GBM [13].

Moreover, it has been also described that glycolysis does not account for the total production of ATP in GBM, suggesting the role of other metabolic routes [9] such us beta oxidation of fatty acids in the mitochondria [14] that requires the endoplasmic reticulum (ER) participation too. ER plays a central role in regulation of the adaptative response needed for a tumoral cell in order to survive to the hostile microenvironment generated as a consequence of the high proliferation rate that includes oxidative stress, nutrient and lipid deprivation and hypoxia [15]. Although the mitochondria and ER were classically thought to work independently, electron microscopy ultrastructural studies already described points of great proximity between the two organelles that made researchers suspect an interrelation between them. These points, known as mitochondria-ER contact sites (MERCS) are sites of close proximity between both organelles where several different proteins are recruited. Among these proteins is the mitochondrial membrane voltage dependent anionic channel (VDAC) that interacts with the Inositol trisphosphate receptor (IP3R) of the ER through the chaperone GPR75, being responsible for the transfer of calcium from the ER to the mitochondria [16]. This calcium is essential for the activity of dehydrogenases of the Krebs cycle, the production of energy and consequently cell survival [17]. Of interest, a recent electron microscopy ultrastructural study has described differences at the level of MERCS between GICs and differentiated tumor cells [18], suggesting an important role of ER-mitochondria interactions in the biology of GBM cells.

We hypothesize that there are differences at the level of ER and mitochondrial functionality between GICs and their differentiated counterparts that plays a central role in regulation of glucose metabolism and that these differences are key to maintaining the glioma stem cell subpopulation.
2. Materials And Methods

2.1. Cell culture and reagents.

Neurospheroid cultures were established from acute cell dissociation of human glioblastoma post-surgical specimens and maintained in DMEM/F12 medium supplemented with B27 (Invitrogen, Carlsbad, CA), EGF and bFGF (20 ng/ml each, Sigma-Aldrich, St Louis, MO) according to the procedures described elsewhere [19, 20]. Neurospheroid cultures display a GICs phenotype (self-renewal, proliferation, expression of stem cell markers, pluripotency, and ability to form tumors in vivo).

Cell culture reagents were purchased from Sigma (Sigma Chemical Co., St Louis, MO, USA) except for FBS, which was obtained from Gibco (Invitrogen Life Technologies, Spain). Culture flasks and dishes were acquired from Fisher Scientific (Madrid, Spain). All other reagents were purchased from Sigma (Sigma Chemical Co., St Louis, MO, USA), unless otherwise indicated.

2.2. Self-renewal assessment.

Self-renewal was determined by the limiting dilution assay, which indicates the number of cells from a primary NS that are needed to form a secondary NS. For this experiment, primary neurospheres were treated overnight with different drugs and then counted with an automatic cell counter and seeded in 96 well plates at dilutions that ranged from 100 cells/well to 1 cell/well. After 7 days of culture, each well was examined for the formation of tumor spheres. Data were analyzed using the web-based tool “ELDA” (extreme limiting dilution analysis) (http://bioinf.wehi.edu.au/software/elda/).

2.3. Evaluation of cell viability.

Cell death was determined by means of Trypan blue exclusion assay. Trypan blue uptake is indicative of irreversible membrane damage preceding cell death, giving as a result a blue staining in non-viable cells. For these assays, cells were seeded in 12-well plates at a density of $10^4$ cells/ml. After treatments, cells were harvested and resuspended in 400 µl of PBS and 100 µl of 0.4% (w/v) trypan blue solution. The number of cells and the percentage of viable and non-viable cells were determined using an automatic cell counter (Countess™ 3, Invitrogen Life Technologies, Spain).

For drug combination studies, cell visibility was evaluated using a colorimetric assay, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, cells were seeded onto 96-well plates and once the treatments were completed, 10 µl of a MTT solution in PBS (5 mg/mL) were added. After 4 hours of incubation at 37 ºC, one volume of the lysis solution [sodium dodecyl sulphate (SDS) 20% and dimethylformamide pH 4.7, 50%] was added. The mixture was incubated at 37 ºC overnight and the samples were measured in an automatic microplate reader (µQuant, Bio-Tek Instruments, Inc., Winooski, VT, USA) at the wavelength of 540 nm.

2.4. Measurement of glucose uptake using 2-NBDG.
Glucose uptake activity was measured using a fluorescent D-glucose analogue 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG). Cells were seeded in 6-well plates at a density of $10^4$ cells/ml and treated with the different compounds for 24h. After treatments, cells were collected and incubated with 10µM 2-NBDG for 35 min. Fluorescence was measured in a microplate fluorimeter FLX-800 (Bio-Tek Instruments, Inc., Winooski, VT, USA) at an excitation wavelength of 467 nm and an emission wavelength of 542 nm. The obtained fluorescence was normalized with total number of cells determined using an automatic cell counter (Countess™ 3, Invitrogen Life Technologies, Spain).

2.5. Determination of mitochondrial calcium levels.

Cells were seeded in 6-well plates at a density of $10^4$ cells/ml. After treatments, cells were collected and incubated with a fluorescent probe specific for mitochondrial Ca\(^{2+}\) [3 µM Rhod-2 AM] for 30 min at 37°C. The fluorescence signal from these cells was measured using a microplate fluorimeter FLX-800 (Bio-Tek Instruments, Inc., Winooski, VT, USA) at an excitation and emission wavelength of 552 nm and 581 nm respectively. The obtained fluorescence was normalized with total number of cells using an automatic cell counter (Countess™ 3, Invitrogen Life Technologies, Spain).


The fluorescent probe Rhodamine 123 was used to monitor the electrochemical gradient in mitochondria ($\Delta \Psi_m$). Cells were seeded in 6-well plates at a density of $10^4$ cells/ml and treated with the different compounds for 24h. After treatments, cells were collected and incubated with 1µg/ml Rhodamine 123 in serum-free medium for 30 min at 37°C. Fluorescence was measured using a microplate fluorimeter FLX-800 (Bio-Tek Instruments, Inc., Winooski, VT, USA) at an excitation and emission wavelength of 488 nm and 515 nm, respectively. The obtained fluorescence was normalized with total number of cells using an automatic cell counter (Countess™ 3, Invitrogen Life Technologies, Spain).

2.7. Evaluation of intracellular peroxides.

The fluorescent probe DCFH-DA was used to monitor intracellular peroxides. Cells were seeded in 6-well plates at a density of $10^4$ cells/ml and treated with the different compounds for 24h. After treatments, cells were collected and incubated with 10µM DCFH-DA in serum-free medium for 30 min at 37°C. Fluorescence was measured using a microplate fluorimeter FLX-800 (Bio-Tek Instruments, Inc., Winooski, VT, USA) at an excitation and emission wavelength of 485 nm and 530 nm, respectively. The obtained fluorescence was normalized with total number of cells using an automatic cell counter (Countess™ 3, Invitrogen Life Technologies, Spain).


Cells were seeded in 24-well plates at a density of $10^4$ cells/ml. Determination of LDH activity was accomplished following specifications of the lactic dehydrogenase based In Vitro Toxicology Assay Kit (Sigma-Aldrich, St Louis, MO, USA). Absorbance was determined using an automatic microplate reader.
(µQuant; Bio-Tek Instruments, Inc., Winooski, VT, USA) at 490 nm and then the obtained data were relativized with total protein concentration.

### 2.9. Western Blot.

For protein expression analysis, cells were lysed in ice-cold lysis buffer (150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% v/v Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 µg/mL leupeptin, 2 µg/ml aprotinin, 1 µg/ml pep-statin-A, 110 nM NaF, 1 mM PMSF, 20 mM Tris–HCl pH 7.5). Thirty micrograms of total protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Amersham Bioscience, Pittsburgh, PA, USA). Blots were incubated overnight at 4 ºC with appropriate antibodies (Supplemental Table 1). Immunoreactive polypeptides were visualized using horseradish peroxidase conjugated secondary antibodies (anti-rabbit or anti-mouse IgG peroxidase conjugated 1:4000; Santa Cruz Biotechnology, Dallas, TX, USA) and enhanced-chemiluminescence detection reagents (Merck Millipore) following manufacturer-supplied protocols.

### 2.10. Real Time Quantitative PCR (Q-RT-PCR).

Total RNA was extracted from cells using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma Chemical Co., St Louis, MO, USA). cDNA was made by reverse-transcribing 1 µg of total RNA using Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit. Quantitative analysis of CD133, SOX 2, OCT3/4, and NANOG levels was performed by the SYBR Green real time PCR method using Green PCR Core Reagents (ABI systems; Foster City, CA) in an AB7700 Real-Time System (ABI systems; Foster City, CA). The primers used are presented in Supplemental Table 2. Each sample was tested in triplicate, and relative gene expression data was analyzed by means of the 2−ΔCT method.

Evaluation of the levels of expression of glucose metabolism related genes, RNA was converted to cDNA using the RT2 First Strand Kit (Qiagen), and RT-PCR was performed using the Qiagen Glucose Metabolism RT2 Profiler PCR Array with the RT2 SYBR Green qPCR Mastermix (Qiagen) on an AB7700 Real-Time System (ABI systems; Foster City, CA). Data analysis was performed as described by the manufacturer.

### 2.11. Data Analysis.

Experiments were repeated at least three times, and data were calculated as the average ± Standard Error. Significance was tested by t-test when two groups were compared, while one-way ANOVA followed by a Student-Newman-Keuls multiple range test was used to multiple range test. Statistical significance was accepted when p ≤ 0.05.

### 3. Results

Differences between stem cells and differentiated counterparts were compared in two different glioblastoma patients (GBM grade IV) derived neurospheroid cultures, GIC-A and GIC-B. As expected, after 10 days of culture in a serum containing medium, both display a differentiated phenotype with an astrocyte-like morphology and numerous processes forming well-delineated bushy territories
Further, mRNA expression levels of several stem cell markers such as Sox2, Oct4, Nanog, and CD133 show a decrease after culture in serum-containing medium (Supplemental Fig. 1B). Decrease of Sox2, a transcription factor whose activity has been described to be essential for glioblastoma stem cells [21] was also demonstrated at the protein levels, together with an increase of GFAP expression—a marker of differentiation—(Supplemental Fig. 1C).

3.1. Glioma initiating cells are dependent on mitochondrial metabolism.

Glucose metabolism in GICs is still unclear since both aerobic glycolysis and mitochondrial dependent glucose metabolism have been described in the literature [13]. To assess this discrepancy, we have evaluated glucose uptake, mitochondrial membrane potential and LDH activity (main enzyme in the production of lactate during aerobic glycolysis) in our experimental model. As shown in Fig. 1, we found a decrease in glucose uptake (Fig. 1A) and a disruption in mitochondrial membrane potential—as determined by rhodamine 123 fluorescence—(Fig. 1B) after differentiation of cells (10 days of serum containing medium incubation), while LDH activity increases under those culture conditions (Fig. 1C). Moreover, a decrease in intracellular reactive oxygen species (mainly produced in the mitochondria) also occurs during glioma stem cells differentiation (Fig. 1D). All these data seem to indicate that under differentiated conditions, GICs change their metabolism, going from a greater dependence on mitochondria to a greater dependence on aerobic glycolysis. In this sense evaluation of the expression of 84 glucose metabolism related genes using the Qiagen Glucose Metabolism RT2 Profiler PCR Array (Fig. 1E) demonstrated a decrease in the expression of 36 of those genes after differentiation in serum containing medium, including some key genes such as HK2 (hexokinase 2) or PDH (pyruvate dehydrogenase). Only one of the studied genes—TPI—, that encodes for the triosephosphate isomerase—one of the key glycolytic enzymes—, increased the expression in differentiated glioma cells. Downregulation of HK2 and PDH were also confirmed at the protein level (Fig. 1F).

According to the previous results, disruption of mitochondrial electron transport chain by rotenone resulted in the induction of GICs cell death with a significantly fewer effect on the population of differentiated cells (Fig. 2A-B). In the same line, inhibition of lactate dehydrogenase by oxamate haven’t a significant effect on GICs while it induced cell death in differentiated cells (Fig. 2A-B), reinforcing the idea that after differentiation cells become more dependent on aerobic glycolysis than mitochondrial metabolism. Moreover, disruption of ETC by rotenone also resulted in a decrease of self-renewal (Fig. 2C) that correlates with a decrease in the expression of the stem cell marker Sox2 (Fig. 2D) which has been described to play a key role in glioblastoma cell stemness and tumor propagation [22].

3.2. Differences in mitochondrial metabolism between GICs and differentiated tumor cells are related to calcium flux.
It is well known that calcium uptake by the mitochondria is essential for the activity of dehydrogenases of the Krebs cycle and so the production of energy at the mitochondria [23]. In this sense, we found a decrease in the calcium levels inside the mitochondria (Fig. 3A), which can be implicated in the decrease of the mitochondrial activity observed. A decrease in the expression of voltage-dependent anion channel (VDAC), the main carrier of Ca\(^{2+}\) in the outer mitochondrial membrane, was also observed after differentiation, which can be responsible of the observed decrease in mitochondrial calcium (Fig. 3B). Moreover, treatment of cells with an intracellular calcium chelator, BAPTA, resulted in the induction of cell death in the GICs subpopulation without any effect on their differentiated counterparts (Fig. 3C) and also a decrease in the self-renewal capability of GICs (Fig. 3D), indicating an essential role of cellular calcium flux in the maintenance of GICs.

As stated before [23], among the numerous functions that calcium plays in the mitochondria is the regulation of the dehydrogenases of the Krebs cycle and so it is closely related with cellular energy metabolism. In this regard we found that treatment of GICs with rotenone induces a disruption of mitochondrial membrane potential, as determined by a decrease in Rhodamine 123 fluorescence (Fig. 4A) and a slight but significant increase in LDH activity (Fig. 4B) that is accompanied by a decrease in the mitochondrial calcium levels (Fig. 4C). In the same way, BAPTA treatment, although it was capable of inducing an increase in glucose uptake (Fig. 4D), it also induces a disruption of mitochondrial membrane potential, as determined by a decrease in the Rhodamine 123 fluorescence in GICs (Fig. 4E), reinforcing the idea of a close relationship between calcium flux to the mitochondria and glucose metabolism in GICs.

### 3.3. ER-mitochondria interaction is determinant for GICs metabolism and maintenance.

In agreement with data obtained after BAPTA treatment, incubation of GICs with thapsigargin, a potent inhibitor of the ion transport activity of sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPases (SERCA), that disrupt calcium homeostasis at the ER -the main calcium reservoir in the cell- also induced cell death (Fig. 5A). Moreover, thapsigargin treatment of GICs also induces a decrease in mitochondrial calcium (Fig. 5B) that is accompanied by an increase in LDH activity (Fig. 5C) suggesting that disruption of calcium homeostasis at the ER results in an alteration of mitochondrial calcium that is probably related to a swift from mitochondrial metabolism to aerobic glycolysis.

Since thapsigargin, due to its action on SERCA, is commonly used as an ER stress inducer, these results also point out the possible relevance of ER homeostasis in GICs maintenance. Of interest, we found a decrease in the expression of Bip, a central regulator for ER stress [24], after serum induced GICs differentiation (Fig. 5D). Moreover, we have also found a decrease in the expression of ERO1\(\alpha\), PDI and the ER calcium binding protein calnexin (Fig. 5E) under differentiation culture conditions. ERO1\(\alpha\) and PDI have been described to play a crucial role in ER calcium homeostasis and calcium transfer to the mitochondria [25].
The use of modulators of ER activity also reflected differences between GIC and their differentiated counterparts. Thus, treatment of cells with tunicamycin, another ER stress inducer, also resulted in GICs death without any cytotoxic effect in their differentiated counterparts cells (Fig. 6A), while treatment of cells with the chemical chaperone 4-phenyl butyric acid (4-PBA), that attenuates ER stress, had no cytotoxic effects on GICs or differentiated (data not shown) Moreover, tunicamycin treatment also induces an increase in glucose uptake that correlates with a decrease in rhodamine 123 fluorescence (Fig. 6B-C), as we observed for treatment with BAPTA, indicating that this glucose is probably derived to a metabolic pathway other than mitochondrial. Tunicamycin treatment also induces a decrease in self-renewal capability in GICs (Fig. 6D).

All together these data suggest an important role of ER in the regulation of calcium flux and glucose metabolism in GICs. It is important to notice that mitochondrial calcium influx represents one of the main functions of ER-mitochondria connections through MERCS [16]. In this regard, it is well known that the tight connection between VDAC1 and the IP3 receptor (IP3R) through the chaperone GPR75 represents the main mechanism of ER to mitochondria calcium influx. Of interest, although we found a decrease in the expression of IP3R (Fig. 7A) after serum induced differentiation, we also found a decrease in the expression of GRP75, both at mRNA (Fig. 7B) and protein levels (Fig. 7A), in addition to the decrease in VDAC expression that we had already observed. The use of a chemical GRP75 inhibitor, MKT-707, revealed that inhibition of this protein was toxic for GICs (Fig. 7C). MKT-077 treatment also induced a disruption of the mitochondrial membrane potential on GICs, as reflected by a decrease in rhodamine 123 fluorescence (Fig. 7D), reinforcing the idea of a key function of this ER-mitochondria connections in the regulation of GICs metabolism, and also potentiates the effect of temozolomide on GICs (Fig. 7E) - current treatment for malignant gliomas- opening the possibility of using this protein as a target for the development of new therapies.

4. Discussion

Our study indicated that glioblastoma cells are heterogeneous in their metabolic phenotypes, being GICs more dependent on oxidative phosphorylation at the mitochondria than their differentiated progeny. This distinct metabolic state seems to be related to ER homeostasis that affects ER-mitochondrial contact sites functioning.

Genetic alterations and environmental modifications, such as hypoxia, converge in one of the traits that define tumor cells and that are in the spotlight for the design of new therapeutic strategies. In fact, metabolic adaptation is considered one of the hallmarks of cancer [8] being aerobic glycolysis or the Warburg effect the main change in cancer cells. Thus, cancer cells move from oxidative phosphorylation (OXPHOS) as a way of obtaining their energy towards lactate production, even at normal oxygen concentrations. However, there are relatively few studies and quite a few discrepancies in relation to the metabolic pathways used by cancer stem cells in general and GICs in particular. Some studies indicate that CSCs have a distinctive metabolic phenotype compared with tumor bulk cells, although there is so far no consensus on this. Thus, both the preferential use of aerobic glycolysis and the mitochondrial
oxidative metabolism have been described [13]. Trying to address this discrepancy, our results showed that GICs use mitochondrial metabolism preferably and that this metabolic pathway decreases after differentiation to tumor bulk cells. Although we found a decrease in glucose uptake - which can be considered contrary to what happens in the Warburg effect - glioma cells increase LDH activity in correlation to an alteration of mitochondrial activity when differentiated. Moreover, GICs are more sensitive to the inhibition of electron transport chain by rotenone and less sensitive to LDH inhibition by oxamate compared to their differentiated counterparts. These results are in agreement with other authors that described a decrease of stemness in cancer stem cells from different tumors, such us breast or prostate cancer, after inhibition of complex I of the ETC [26, 27]. It must be considered that several studies carried out on different types of tumors seem to indicate that the metabolic phenotype of cancer stem cells can be modified depending on the state of differentiation, tumor microenvironment, or expression of certain oncogenes, which could explain divergence results already published, even within the same tumor type [28].

Mitochondrial bioenergetics is largely controlled by extra mitochondrial events whose activity are frequently altered in cancer such us calcium homeostasis. Mitochondria can act both as a reservoir of Ca^{2+} and as an effector that utilize Ca^{2+} to regulate cell survival, proliferation, redox state and metabolic changes [29]. This mitochondrial Ca^{2+} homeostasis requires an efficient interplay between endoplasmic reticulum (ER), where most intracellular Ca^{2+} is stored, and mitochondria through MERCS [30, 31]. Thus, controlled raises in matrix Ca^{2+} concentration have important metabolic effects, as Ca^{2+} enhances the activity of mitochondrial dehydrogenases of the TCA cycle, IDH and αKGDH, and of PDH [31]. The decrease in mitochondrial calcium that we observed after GICs differentiation could be responsible, at least in part, for the distinct metabolic phenotype observed between GICs and their differentiated progeny. In fact, we found that alterations in calcium homeostasis using calcium chelators not only resulted toxic for the GICs subpopulation, decreasing also self-renewal capacity, but also induced changes in glucose metabolism such us an increase in glucose uptake and a disruption in mitochondrial membrane potential. Although these results may appear contradictory, the fact that an increase in glucose up-take in GICs dos does not correlate to an increase in mitochondrial activity could be explained by the fact that use of that glucose might be derived to other metabolic pathways. As an example, it has been described that quiescent breast CSCs have a high metabolic rate of the pentose phosphate pathway (PPP), which favors the generation of reducing power (NADPH), essential for the maintenance of the state cellular redox [32].

The decrease in the expression of Bip, central regulator of ER stress responses, after GICs differentiation, together with the fact that we observed similar effects in cell viability, self-renewal and glucose metabolism when using ER stress inducers compared to calcium chelators, seems to indicate that ER homeostasis also plays a key role in GICs maintenance and metabolism. Moreover, we also observed a decrease in the expression of ER01α, PDI and the ER calcium binding protein calnexin after differentiation. ER01α-PDI have been described to be enriched at the MERCS interface and to play a
crucial role in calcium flux from ER to mitochondria. Thus, downregulation of Ero1α inhibits mitochondrial Ca\(^{2+}\) fluxes and modifies the activity of mitochondrial Ca\(^{2+}\) uniporters [25].

In this sense, it is important to notice that mitochondrial calcium influx represents one of the main functions of ER-mitochondria connections [16], being the IP3Rs-Grp75-VDACs complex the basis for the mitochondrial Ca\(^{2+}\) transfer in MERCS [33]. In this regard, we found a decrease in VDAC expression in differentiated cells that can be responsible for the observed decrease in mitochondrial calcium level. The relevance of VDAC expression in glioblastoma has been already described before. Thus, inhibition of VDAC expression by siRNA has been described to inhibit GBM growth and to reduce angiogenesis, invasiveness and stemness [34]. We also found a decrease in the expression of Grp75, also known as mortalin, after GICs differentiation. Moreover, the use of a chemical Grp75 inhibitor revealed an essential role of this protein in GICs survival, chemoresistance and mitochondrial metabolism maintenance. This protein has been described to be enriched in a large variety of cancers and it is also considered to act as a regulatory factor in the maintaining the stemness of the cancer stem cells [35]. Thus, it has been described that mortalin is able to upregulate the activity of some cancer stem cells signaling pathways such as Wnt/GSK3β/β-catenin in breast and colorectal cancer [36, 37]. Moreover, upregulation of mortalin expression in correlation with malignant progression in brain tumors has been described several years ago [38]. Combination of Grp75 inhibitor and temozolomide, current chemotherapeutic treatment for malignant gliomas, resulted in an increased GICs death, suggesting that Grp75, and so the ER-mitochondria connection through IP3Rs-Grp75-VDACs complex, is also important for chemoresistance of this cancer stem cell subpopulation and could perhaps be taken into account as a target for the development of new therapies.

Declarations

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Noelia Puente-Moncada and Marco Alvarez-Vega; Writing original draft: Isaac Antolin, Carmen Rodriguez and Vanesa Martin; Writing-review & editing: Federico Herrera, Jezabel Rodriguez-Blanco, Carmen Rodriguez and Vanesa Martin.

**Ethics approval:** the study was conducted in accordance with the Declaration of Helsinki and approved by Clinical Research Ethics Committee of the Principality of Asturias (protocol code 2021.008 approved on January 22nd, 2021).

**References**


Figures

Figure 1
GICs relay more on mitochondrial metabolism that their differentiated progeny. Glucose uptake (A), mitochondrial membrane potential -as rhodamine 123 fluorescence per cell- (B), LDH activity (C) and intracellular peroxides (D) were determined in neurosphere cultures and their differentiated counterpart (10 days of culture in serum containing medium). * p ≤ 0.05 versus GIC. E, mRNA expression levels of glucose metabolism related genes determined by real-time quantitative PCR. Only those genes with a decrease in their expression of at least half or an increase in their expression of at least double have been represented. Dashed line indicates basal level of expression. ACO1, Aconitase 1, soluble; AGL, Amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase; ALDOA, Aldolase A, fructose-bisphosphate; ALDOC, Aldolase C, fructose-bisphosphate; BPGM, 2,3-bisphosphoglycerate mutase; CS, Citrate synthase; DLAT, Dihydrolipoamide S-acetyltransferase; DLD, Dihydrolipoamide dehydrogenase; DLST, Dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex); ENO3, Enolase 3 (beta, muscle); G6PC3, Glucose 6 phosphatase, catalytic, 3; G6PD, Glucose-6-phosphate dehydrogenase; GALM, Galactose mutarotase (aldose 1-epimerase); GBE1, Glucan (1,4-alpha-), branching enzyme 1; GSK3A, Glycogen synthase kinase 3 alpha; GSK3B, Glycogen synthase kinase 3 beta; GYS1, Glycogen synthase 1 (muscle); H6PD, Hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase); HK2, Hexokinase 2; IDH1, Isocitrate dehydrogenase 1 (NADP+), soluble; IDH2, Isocitrate dehydrogenase 2 (NADP+), mitochondrial; IDH3A, Isocitrate dehydrogenase 3 (NAD+) alpha; IDH3B, Isocitrate dehydrogenase 3 (NAD+) beta; PC, Pyruvate carboxylase; PCK1, Phos-phoenolpyruvate carboxykinase 1 (soluble); PCK2, Phosphoenolpyruvate carboxykinase 2 (mitochondrial); PDHA1 Pyruvate dehydrogenase (lipoamide) alpha 1; PDHB, Pyruvate dehydrogenase (lipoamide) beta; PDK1, Pyruvate dehydrogenase kinase, isozyme 1; PDP2, Pyruvate dehydrogenase phosphatase catalytic subunit 2; PDPR, Pyruvate dehydrogenase phosphatase regulatory subunit 2; PGAM2, Phosphoglycerate mutase 2 (muscle); PHKA1, Phosphorylase kinase, alpha 1 (muscle); PHKG1, Phosphorylase kinase, gamma 1 (muscle); PRPS1, Phosphoribosyl pyrophosphate synthetase 1; PRPS1L1, Phosphoribosyl pyrophosphate synthetase 1-like 1; TPI1, Triosephosphate isomerase 1. F, protein expression of HK2 and PDH in both GICs and their differentiated counterparts. GAPDH expression has been used as loading control. Dashed line represents levels of expression of GICs. * p ≤ 0.05 versus GIC.
Figure 2

Mitochondrial metabolism is essential for GICs maintenance. A-B, cell death determined after treatment of GICs and their differentiated counterparts with 500nM rotenone (ETC inhibitor) or 20mM oxamate (LDH inhibitor) for 48h. * p ≤ 0.05 versus GIC. C, in vitro self-renewal limiting dilution assay after overnight treatment with 500nM rotenone was performed for the two different GIC lines. After treatment, cells were seeded at dilutions that ranged from 100 cells/well to 1 cell/well and the number of cells that
are needed to form a secondary NS was determined after 10 days in culture. Estimated stem cell frequency for each experimental group was determined using a web-based tool (ELDA, http://bioinf.wehi.edu.au/software/elda/). D, decrease in the protein expression of sox2 after treatment of GICs with 500nM rotenone for 24h. Actin expression has been used as loading control.

**Figure 3**

A: Graph showing mitochondrial calcium (RFU) for GIC-A and GIC-B with and without FBS.

B: Western blot images showing VDAC and GAPDH expression for GIC-A and GIC-B under FBS conditions.

C: Graph showing % Cell death for GIC-A and GIC-B with different treatments including BAPTA and FBS.

D: Graphs showing number of secondary neurospheres for GIC-A and GIC-B with varying cell numbers per well.

<table>
<thead>
<tr>
<th>Group</th>
<th>GIC-A</th>
<th>GIC-B</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>9.65</td>
<td>8.32</td>
</tr>
<tr>
<td>BAPTA</td>
<td>15.77</td>
<td>18.54</td>
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</table>
Modulation of calcium flux by differentiation in GICs. (A) mitochondrial calcium levels were determined in neurosphere cultures and their differentiated counterpart (10 days of culture in serum containing medium). * p ≤ 0.05 versus GIC. B, decrease in the protein expression of VDAC after GICs differentiation. GAPDH expression has been used as loading control. C, cell death determined after treatment of GICs and their differentiated counterparts with 10µM BAPTA (intracellular calcium chelator) for 48h. * p ≤ 0.05 versus its own control (GIC or differentiated counterpart). D, in vitro self-renewal limiting dilution assay after overnight treatment with 10µM was performed for the two different GIC lines. After treatment, cells were seeded at dilutions that ranged from 100 cells/well to 1 cell/well and the number of cells that are needed to form a secondary NS was determined after 10 days in culture. Estimated stem cell frequency for each experimental group was determined using a web-based tool (ELDA, http://bioinf.wehi.edu.au/software/elda/).

Figure 4
Calcium flux and mitochondrial metabolism are close related in GICs. Mitochondrial membrane potential - as rhodamine 123 fluorescence per cell- (A), LDH activity (B) and mitochondrial calcium (C) determined after treatment of GICs with 500nM rotenone for 24h. * p ≤ 0.05 versus untreated cells. Glucose uptake (D) and mitochondrial membrane potential (E) were determined in GICs after treatment with 10µM BAPTA for 24h. * p ≤ 0.05 versus control group (vehicle-treated group).

**Figure 5**

ER homeostasis plays a key role in GICs maintenance and metabolism. Cell death (A), mitochondrial calcium (B) and LDH activity (C) determined after treatment of GICs with 5µM thapsigargin for 48h. * p ≤ 0.05 versus control group (vehicle-treated group). D, decrease in the protein expression of Bip after GICs differentiation. GAPDH expression has been used as loading control. E, expression levels of ER related proteins (ERO1α, PDI, calnexin and IP3R) after differentiation. GAPDH expression has been used as loading control. Dashed line represents levels of expression of GICs. * p ≤ 0.05 versus GIC.
Figure 6

ER homeostasis plays a key role in GICs maintenance and metabolism. A, cell death determined after treatment of GICs and their differentiated counterparts with 500ng/ml tunicamycin (ER stress inducer) for 48h. * p ≤ 0.05 versus its own control (GIC or differentiated counterpart). Glucose uptake (B) and mitochondrial membrane potential (C) were determined in GICs after treatment with 500ng/ml tunicamycin for 24h. * p ≤ 0.05 versus control group (vehicle-treated group). D, in vitro self-renewal limiting dilution assay after overnight treatment with 500ng/ml tunicamycin was performed for the two different GIC lines. After treatment, cells were seeded at dilutions that ranged from 100 cells/well to 1 cell/well and the number of cells that are needed to form a secondary NS was determined after 10 days in culture. Estimated stem cell frequency for each experimental group was determined using a web-based tool (ELDA, http://bioinf.wehi.edu.au/software/elda/).
Figure 7

Grp75 is essential for GICs maintenance. A, protein expression of Grp75 and IP3R after GICs differentiation (10 days of culture in serum containing medium). B, decrease of mRNA expression of Grp75 after GICs differentiation. C, cell death determined after treatment of GICs and their differentiated counterparts with the Grp75 inhibitor MKT-077 (1 to 10µM) for 48h. * p ≤ 0.05 versus GIC. D, mitochondrial membrane potential were determined in GICs after treatment with 1µM MKT-077 for 24h. *
p ≤ 0.05 versus control group (vehicle-treated group). E, effect of combination of temozolomide and MKT-077 (500:1 constant ratio) on GICs cell viability as determined by MTT assay. * p ≤ 0.05 versus temozolomide alone.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementalfigure1.docx
- Supplementaltable1.docx
- Supplementaltable2.docx