Evaluation of miRNA Expression in Glioblastoma Stem-like Cells: a Comparison Between Normoxia and Hypoxia Microenvironment

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Research Article

Keywords: Glioblastoma, stem-like cells, miRNAs, hypoxia microenvironment

Posted Date: July 8th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-681014/v1>

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Abstract

Purpose: Glioblastoma is one of the most aggressive and incurable brain tumors whose progression is driven in part by a small sub population of cells termed as glioblastoma stem cells responsible for the tumor's low therapy efficacy. Hypoxia, a common phenomenon in glioblastoma also promotes the maintenance and the expansion of the stem cell population whose survival is aided by miRNAs.

Methods: GBM stem-like cells cultures were isolated and the relationship between the microenvironments and the *in vitro* "stemness" of the cells was investigated by evaluating the expression of miRNAs and selected genes.

Results: We found miR-128a-3p, 34-5p and 181a-3p to be down-regulated while miR-17-5p and miR-221-3p to be up-regulated in the stem-like cells. When a comparison was made between the stem-like cells cultured under normoxia and hypoxia, a fold down-regulation difference of 3.5 to 5, 2 to 5 and 2 to 4 for miR-34-5p, 128a-3p and 181a-3p was observed respectively and a fold upregulation of 3.5 to 4 and 2.5 to 4 was observed for miR-221-3p and 17-5p respectively. There was an increased expression of HIF-1/2, SOX2, OCT4, VEGF, GLUT-1, BCL2 and survivin under hypoxia.

Conclusion: Hypoxia enhanced the expression of several tumor stem cell signature genes, involved in the regulation of stemness, metabolism, angiogenesis and anti-apoptotic property. Most importantly, the miRNA dysregulation in the stem-like cell population adds another layer of gene expression associated with gliomagenesis and maintenance of CSC pointing to new signaling mechanisms whose disruption can be used to successfully target this crucial subpopulation.

1. Introduction

Glioblastoma (GBM) is the most common and lethal cancer of the adult brain, remaining incurable with a median survival time of only 15 months (Stupp et al. 2009; Ostrom et al. 2014). Contributing to its poor prognosis are numerous therapeutic challenges among them being the tumor's aggressive growth nature, chemo and radio-resistance and recurrence attributed mainly to the presence of a small population of tumor cells within the GBM with self-renewing and tumor-propagating capacity (Stupp et al. 2009; Campos et al. 2016; Sharifzad et al. 2019). This sub-population of cells is referred to as glioblastoma stem-like cells (GSCs) and only by their eradication can glioblastoma be successfully cured (Chen et al. 2012; Sharifzad et al. 2019). GSCs reside in special niches influenced by microenvironmental factors including hypoxic niches, also common in glioblastoma, which are created from the inefficient blood supply, resulting in hypoxia that subsequently induces pseudo-palisading necrosis (Ishii et al. 2016).

The hypoxia microenvironment has been shown to drive the selection and maintenance of a stem cell phenotype (Semenza 2010; Fidoamore et al. 2016; Ishii et al. 2016). Although some of the effects of hypoxia negatively impact tumor cell growth, they may also lead to hypoxia-driven responses that enhance malignant progression and aggressiveness, ultimately resulting in increased resistance to therapy and poor long-term prognosis (Vaupel 2004). In response to low oxygen, cellular responses to

hypoxia are commonly regulated by the HIFs, a family of transcriptional factors upregulating the transcription of numerous hypoxia-inducible genes. Hypoxia-inducible factors (HIF1 and HIF2) are upregulated and activated and consequently activate genes involved in survival mechanisms and the stem cell regulators (Schito and Semenza 2016; Sharifzad et al. 2019). HIF-1 α is activated in normal neural progenitors, thus limiting its value, in terms of therapeutic targeting while HIF-2 α , is specifically elevated in glioblastoma stem cells, even under modest hypoxic conditions, and practically absent in non-glioma stem cells, and thereby making HIF-2 α an interesting therapeutic target in glioblastomas (Li et al. 2009b; Huang et al. 2016). In an effort to identify new molecular targets for GBM diagnostics and therapeutics, studies have focused on microRNAs (miRNAs) due to their regulatory capacities in normal development and in pathological states such as cancer (Calin and Croce 2006). The biogenesis of miRNAs has been explained in detail in our previous article (Macharia et al. 2019). The miRNAs play important roles in GBM and have been associated with various facets of the selection and promotion process for GSCs (Shea et al. 2016; Macharia et al. 2019). However, the mechanisms underlying the role of miRNAs in the stemness of GSCs are yet to be completely elucidated (Huang et al. 2017). Further study of miRNAs function in CSC may lead to novel treatment strategies for GBM patients and in addressing the therapeutic challenge they present.

Glioblastoma is conventionally investigated *in vitro* by culturing cells as a monolayer (2D culture) or as neurospheres (clusters enriched in cancer stem-like cells) with an oxygen tension of 20%. However, the oxygen tension in GBM *in vivo* ranges from 0.1 to 10% (Muz et al. 2015). The 2D normoxic model fails to take into account the physiologically relevant oxygen tension (Richards et al. 2016; Musah-Eroje and Watson 2019) as well as the interactions within the extracellular matrix which play a role in influencing the tumor's biological properties like proliferation and motility when investigating GBM growth *in vitro* (Gilkes et al. 2014). Neurosphere culture has been regarded as the gold standard model for isolation of the stem cell populations. However, its main limitation is that of allowing cells to form their own niche, where more differentiated cells positioned at the center than on the surface as well as containing a mixed population of cells and a small number of true stem cells (Bez et al. 2003). To circumvent this limitation, conducting experiments under the hypoxia microenvironment as compared to the conventional normoxia microenvironment or the use of 3D culture models that may mimic the intratumor environment more than monolayer cultures may help to give accurate events of GBM biology *in vitro*. As stated previously, the hypoxia microenvironment was used as a potential model to study GSCs in depth. We also explored the 3D culture model as a culture alternative that can mimic the tumor's microenvironment. Therefore, this study investigated the expression levels of a selected list of miRNAs and selected genes in stem-like cells isolated from three types of human glioblastoma cells.

2. Materials And Methods

2.1. Reagents

All the culture media components, DMEM/F12 Dulbecco's Modified Eagle Medium/Nutrient Mixture (#12400-024) and DMEM/F12 Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (#12500-039)

for making the NS34 Neuro Basal medium were supplied by Gibco®; HEPES was supplied by Life Technologies (São Paulo, Brazil) and fetal bovine serum (FBS) was supplied by Invitrogen (Paisley, UK). The growth factors B27 (#1989730), N2 (1969972) and G5 (#1922428) were obtained from Thermo Scientific (São Paulo, Brazil). Penicillin and Streptomycin were purchased from Gibco. Fungizone was purchased from Bristol-Myers Squibb (Princeton, NJ). Glucose was purchased from Merck (Darmstadt, Germany). All culture plates and flasks were obtained from TPP (Trasadingen, Switzerland). Protease and phosphatase inhibitors were supplied by Roche (Indianapolis, IN). Antibodies for Oct-4A (#2840) and SOX2 (#D6D9) were purchased from Cell Signaling Technology (Beverly, MA). The anti-GLUT-1 (#07-1401), anti-HIF-1 α (MAB5382) anti-HIF-2 α (MAB3472) antibodies were purchased from Millipore Corporation (Single Oak drive, CA, USA) Anti-VEGFA (#ab46154) and anti-Survivin (#ab76424) anti-BCL2 (#ab33862) antibodies were purchased from Abcam (Cambridge, MA, USA). The secondary antibodies, conjugated to Alexa Fluor 488(#A-11008, A-21131) and 546 (#A-11010, A-21124) or HRP conjugated anti-mouse (#G-21040) or rabbit (#G-21234) antibodies were purchased from Thermo Scientific (Rockford, USA). PVDF membranes were purchased from Millipore (Billerica, MA). The 4', 6-diamidino-2-phenylindole (DAPI) was obtained from Sigma (MA, USA). 2 $\times$ Laemmli buffer and β -mercaptoethanol were purchased from Bio-Rad (São Paulo, Brazil).

2.2. Cells and culture conditions

The GBM03 and GBM95 glioblastoma cell lines were established and characterized in our laboratory, as previously described (Faria et al. 2006) T98G is an ATCC cell line. The GBM cells were maintained in Dulbecco's modified Eagle's medium (DMEM/F12) supplemented with 10% fetal bovine serum (FBS). Non-adherent cultures of oncospheres were obtained by first growing GBMs cells in serum-free Dulbecco modified Eagle's medium-F12 (DMEM/F12). After reaching semi- confluence, the cells were maintained in serum free NS34 medium made of 5x DMEM/F12 Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (#12500-039), 30% glucose, 200nM Glutamine, 1M Hepes, 7.5% sodium bicarbonate, penicillin streptomycin 100 μ g/mL and the growth factors N2 (100x), B27 (50x) and G5 (100x) as described by (Patru et al. 2010). The cultures were maintained in normoxia chamber set at 37°C in an atmosphere containing 95% air and 5% CO₂ for the normoxic culture and at 1% O₂ in a hypoxia chamber (Thermo scientific) for the hypoxia culture. The undifferentiated cells were separated from differentiated and the cell clusters maintained in NS34. After about 15 days in the culture medium, the cells were evaluated for the expression of the transcription factors and mechanically dissociated for the clonogenicity assay.

2.3. 3D culture model

The 3D culture models were done according to (Tang et al. 2016) using agarose gel. A 24 or 96 well plates were coated with 300 μ L or 100 μ L of 1.5% agarose diluted in 1x phosphate buffered saline (PBS) respectively. The models were frozen for 5 minutes to allow for the agarose to solidify and maintained at room temperature before use. Additionally, 8000 cells were plated in each well of a 24 or a 96 well plate in either in NS34 stem cell media or DMEM/10% FBS media and followed up for 12 days in culture maintained at 37°C temperature at 5% CO₂ and 95% air atmosphere.

2.4. Clonogenicity Assay

After dissociation, the stem-like cells were seeded at a density of 1–2 cells per well in 100µL of NS34 medium in a 96-well plate. This was done for N1, N2 and N3 biological replicates of the same cell lineage. The cells were cultured at 37°C in a humidified 5% CO₂ and 95% air atmosphere overnight. In the following day, the plates were observed for cell growth and the numbers of wells with cells were marked with a tick and those without cells were marked with an X. This data was marked in a custom-made template reflecting the wells in the 96 well plate. When necessary, 10 µL of the NS34 medium was added weekly per well to supplement the old media. Similarly, the wells were counted for sphere formation once a week for the 4 weeks and the number of wells with spheres ticked in the custom-made templates. At the end of the 4 weeks the information was collected and an average done for the N1, N2 and N3. A growth curve was drawn reflecting the self-renewal ability of the cells.

2.5. Immunocytochemistry

Cover slips were put into a 24 well plates and covered with 400µL of 1% polylysine or polyornitin and after incubated at 37°C for 40 minutes. After, the polylysine or polyornitin was removed from the coverslips and washed two times with distilled water. The ready cover slips were left in the fridge in 1% PBS until ready for use. On the day of plating, the PBS was removed and about 20µL of the media containing the oncospheres was put in each well. Incubation was done for 40 minutes at 37°C. After, the media was removed and the coverslips washed once with 1% PBS. Fixing was done using 4% PFA and the rest of the immunocytochemistry procedure proceeded as mentioned above. Samples were incubated with primary antibodies including; anti-Sox2 (Cell signaling, 1:400), anti-Oct4 (Cell signaling, 1:400), anti-HIF-1alpha (Millipore, 1:500), anti-HIF-2alpha (Millipore, 1:500), anti-Glut-1 (Millipore, 1:500), anti-VEGF (Abcam, 1:500), anti-Bcl2 (Abcam, 1:500) and anti-Survivin (Abcam, 1:500). In the following day, the slips were washed with 1% PBS and incubated with secondary antibodies conjugated with Alexa Fluor 488 (goat anti-mouse/rabbit; 1:2000) or Alexa Fluor 546 (goat anti-rabbit; 1:2000) for 2 hours. Cells were imaged using a DMI8 advanced fluorescence microscopy (Leica Microsystems, Germany). The fluorescence intensity of individual cells was measured and analyzed using ImageJ software (NIH, USA).

2.6. qPCR

Total RNA was extracted from the cells using Trizol Reagent (Ambion, Life Technologies) and the PureLink® RNA Mini Kit (Invitrogen, Thermoscientific) following the manufacturer's instructions. Sample RNA purity was estimated using (Nanodrop lite, Thermoscientific) spectrophotometer where a ratio of 1.8 to 2 was considered pure. One microgram of the total RNA, pool RT microRNA primer (custom made) and SuperScript™ III Reverse Transcriptase (SS III, Invitrogen, Life technologies) were used to perform the cDNA synthesis. Quantitative real time PCR (qRT-PCR) was done using Power SYBR green PCR master mix (Applied biosystems, Thermofisher scientific) and custom-made microRNA primers from Intergrated DNA Technologies (IDT). The primers used were designed as follows:

miR-34-5p (Fw: 5'-ACACTCCAGCTGGGTGGCAGTGTCTTAGCT-3';

RT 5'-CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAGACAACC-3',
 miR-128-3p (Fw 5'-ACACTCCAGCTGGGTACAGTGAACCGGTC-3';
 RT 5'-CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAGAAAGAGAC-3'),
 miR-181a-3p (FW 5'-ACACTCCAGCTGGG- ACCATCGACCGUUGAT-3',
 RT 5'-CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAG-GGTACAAT-3'),
 miR-17-5p (Fw 5'-ACACTCCAGCTGGGCAAAGTGCTTACAGTG-3',
 RT 5'-CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAGCTACCTGC-3'),
 miR-221-3p (FW 5'-ACACTCCAGCTGGG-AGCTACATTGTCTGCT 3';
 RT 5'-CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAG-GAAACCCA-3'),
 SNU6 (FW 5'-GCTTCGGCAGCACATATACTAAAAT-3',
 RT 5'-CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAGCGTTCCA-3')

The following primer list was used for the study of the gene expression

FW Human HIF-1 α 5' CTCACAAGATGATGGTGACATG 3'

Rev Human HIF-1 α 5' TTCCTCATGGTCACATGGAT G 3'

FW Human Actin 5' ATGAAGATCAAG ATCATTGCTCCT 3'

Rev Human Actin 5' ACATCTGCTGGAAGGTGGACA 3'

The run was done using CFX96 Real Time System (Bio-Rad). Melt curves were included in every run and the assays were performed in triplicates. Endogenous RNA U6 (RNU6) was used as control for normalization of mature miRNAs while actin for the gene expression. Analysis of the miRNAs expression was calculated using the $2^{-\Delta\Delta C_q}$ method (Livak and Schmittgen 2001). The data was obtained from three independent experiments and analyzed using Graph pad prism software version 5.

2.7. Statistical Analysis

The experimental data are expressed as the mean $\pm$ standard deviation (SD) of three independent replicates. Statistical differences between two groups were evaluated by the Students t test. Those between more than two groups were subjected to analysis of variance (ANOVA) with Dunnett's as a post-test using GraphPad Prism 5.0 statistics software (GraphPad Software, Inc., La Jolla, CA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Neurosphere formation and the clonogenicity assay

The purification of GSCs requires a multistep process that involves testing of the self-renewal, multipotency, and stem-marker expression (Lathia et al. 2015). But neurosphere formation is a primary step in the formation of these stem-like cells (Galli et al. 2004). In this manner, three GBM cell lines (GBM03, GBM95 and T98G) were cultured in NS34 medium and the conversion process was observed over a period of time. The conversion of our GBM cell lines into neurospheres was evident as early as 14 days and the cells had fully formed neurospheres by the end of the third week. The neurospheres formed from GBM03 and GBM95 (Fig. 1A & B) were larger in diameter than the ones observed in T98G (Fig. 1C) which also had a fewer number of neurospheres. The T98G cell line was slower to convert to neurospheres as compared to GBM95 and GBM03 which had already converted by the 14th day (Supplementary Fig. 1). We also performed the clonogenicity assay to examine the ability of a single cell to grow into a large colony through clonal expansion, also a necessary indicator of undifferentiated cancer stem-like state (Ignatova et al. 2002; Singh et al. 2004). The stem-like cells GBM03-O and GBM95-O were seeded at a density of 1–2 cells per well in a 96 well plate in 100µL of NS34 and followed up for a period of four weeks. We were able to observe colony forming ability of both the GBM03-O and GBM95-O cell lines (Fig. 1C) and a sphere size increment along the four weeks of culture (Fig. 1A & B). The GBM03-O had oncospheeres that were larger in diameter as compared to the GBM95-O, demonstrating its higher clonogenic potential (Fig. 1A-C). The clonogenic capacity of T98G has already been reported previously (HONG et al. 2012; Palumbo et al. 2018).

3.2. The expression of miRNAs by the glioblastoma stem-like cells

The miRNAs play important roles in GBM pathogenesis however, the role miRNAs plays in the stemness of GSCs are yet to be completely elucidated (Huang et al. 2017). Studies using actual stem cells as compared to parental cells are warranted as they could add a new layer of directed therapeutic options. Therefore, after conversion of our cell lines into neurospheres, we proceeded to evaluate the miRNA expression between the glioblastoma stem-like cells (GBM-03-O, 95 – O and T98G-O) and their respective parental cells under normoxia conditions. There was a significant downregulation in miR-34-5p, 181a and 128-3p (Fig. 2A-C) and a significant upregulation in miR-17-5p and 221-3p in the actual stem-like cells compared to their respective parental GBMs (Fig. 2D & E). The expression pattern of the miRNAs was maintained as to when the comparison was done between the GBM cell lines compared to a non GBM control cell line (results not shown). Our results suggest that the miRNAs with tumor suppressor properties in GBM (miR-34-5p, 181a and 128-3p) were downregulated also in the stem like cells. Similarly, the miRNAs with the oncogenic properties in GBM (miR-17-5p and 221-3p) were upregulated in the stem-like cells. Specifically, this was about 2 -3-fold increase for GBM-03-O and 95-O) (Fig. 2A-C).

3.3. The effect of hypoxia microenvironment on the morphology of the stem-like cells

The hypoxia microenvironment has been associated with the induction and maintenance of the stem-like cell population and these specialized niches or hypoxic niches are found within the glioblastoma tumor (Ishii et al. 2016; Heffernan and Sirianni 2018). However, most studies investigate neurospheres *in vitro* by culturing cells in oxygen tension of 21%. Therefore we cultured our stem-like cells in hypoxic microenvironment to recapitulate the GBM oxygen tension *in vivo* (Muz et al. 2015). Also, most hypoxia studies are done using GBM as compared to using the actual GSCs. For this, we aimed to evaluate the effect of the hypoxia microenvironment on the actual stem-like cells isolated from the GBMs. We cultured the stem-like cells under hypoxia microenvironments and confirmed their hypoxia acquisition by evaluating the expression of HIF-1/2 α . Results showed expression of HIF-1 α in cells cultured under hypoxia (Fig. 3A2 & C). Traces of HIF-1 α expression could be observed in the inner zones of the neurospheres cultured under normoxia (Fig. 3A1) suggestive of a hypoxic state in the innermost zones of the neurospheres. All our stem-like cells expressed HIF-2 α under both microenvironments, but the expression was upregulated under hypoxia (Fig. 3B1-B2). HIF-2 α is reported to be elevated in glioblastoma stem cells irrespective of the microenvironment (Li et al. 2009b; Huang et al. 2016) which was also confirmed by our results. We aimed to see if hypoxia would induce any morphological changes to the stem-like cells as compared to their counterparts grown under normoxia. We were able to observe that, under hypoxia, the stem-like cells (03-O, 95-O and T98G-O) had bigger spheres in diameter as compared to their counterparts in normoxia. Similarly, as mentioned earlier the 03-O and 95-O have generally larger spheres than T98G-O (Supplementary Fig. 2).

3.3.1. Effect of hypoxia on the expression of miRNAs in the stem-like cells

The miRNAs regulate gene expression including genes related to hypoxia microenvironment (Seroocki et al. 2018). Likewise, hypoxia microenvironment has been shown to influence the biogenesis and expression of several miRNAs including hypoxia related miRNAs (HRMs) as summarized in our previous work at (Macharia et al. 2019). Irrespective, there are limited studies entailing the influence of hypoxia using actual stem cells. For this, we aimed to evaluate the changes in the miRNA expression in our stem-like cells after hypoxia induction. When a comparison of the miRNA expression was made between the stem-like cells (03 – 0, 95 – 0 and T98G-0) cultured under hypoxia conditions to normoxia, a fold down-regulation difference of 3.5 to 5, 2 to 5 and 2 to 4 was observed respectively for miR-34-5p, 128a-3p and 181a-3p (Fig. 4A-C) while a fold change upregulation of 3.5 to 4 and 2.5 to 4 was observed for miR-221-3p and 17-5p respectively (Fig. 4D & E). Hypoxia influenced a further downregulation and upregulation.

3.3.2. Hypoxia and the *in-vitro* stemness

The purification of GSCs requires a test for the stem-marker expression that include the transcription factors (Lathia et al. 2015). However, if hypoxia microenvironment drives the reprogramming and the

maintenance of a stem cell phenotype (Ishii et al. 2016; Semenza 2010; Fidoamore et al. 2016), the transcription factors should be upregulated under such microenvironment. To test this hypothesis, we evaluated whether culturing a stem-like cell under hypoxia, would induce further changes in the expression of the transcription factors SOX2 and OCT4. There was an upregulated expression of SOX2 and OCT4 under hypoxia (Fig. 5A2 & B3). Additionally, the expression of SOX2 and OCT4 was present and furthermore, suggestively stronger in the inner zone of the neurospheres cultured under normoxia (Fig. 5A1 & B1). The zones were less visible in the stem-like cells cultured under hypoxia where the expression of SOX2 and OCT4 was equally distributed in the oncospheres and upregulated. This observation suggests that hypoxia microenvironment reprograms the neurospheres to a true stem cell phenotype (Fig. 5A & B).

3.3.3. Evasion of apoptosis and metabolic reprogramming of the stem-like cells

Resisting cell death and metabolic reprogramming are among the hallmarks of cancer (Hanahan and Weinberg 2011; Ward and Thompson 2012). We aimed to evaluate the effect of hypoxia microenvironment on the expression of the anti-apoptotic proteins (Bcl2 and survivin) and GLUT-1 associated with altered metabolism. Our stem like cells expressed these genes under normoxia (Fig. 6A1, B1 & C1). These results were suggestive of an increased apoptotic escape and altered metabolism. Hypoxia microenvironment too did not induce cell death as the Bcl2 and survivin were further enhanced by a 2 to 3 fold increase in the same microenvironment (Fig. 6A2 & B2) similar to GLUT-1 (Fig. 6C2). Therefore, our stem-like cells had altered apoptosis and metabolism and hypoxia induced further differences of expression induced by the microenvironment (Fig. 6A-C).

3.4. The 3D culture model as a culture alternative that can mimic the tumors microenvironment

Three-dimensional culture models of the tumor microenvironment are powerful options for studying cellular interactions (Heffernan and Sirianni 2018). It has been hypothesized that the 3D culture system is a closer and an economical culture model recapitulating microenvironment of the tumors *in vivo* ((Bez et al. 2003; Musah-Eroje and Watson 2019). In this manner, we cultured GBM03 and T98G parental cells under a modified 3D model using the stem cell media NS34 or the conventional culture media DMEM with 10% FBS and followed their growth for up to 12 days. It was observed that the two cell lines were able to form spheroids as early as the 4th day after the culture that was fully formed by the 12th day. For the cells cultured in the 96 well plate, the spheroids formed irrespective of the culture medium used. However, the spheroids cultured using the NS34 medium were suggestively slightly larger in diameter. The spheroids in DMEM/FBS on the other hand had rounded edges (Fig. 7A1 & A2). For the cells cultured in 24 well plate, most of the spheroids were slightly larger in diameter in DMEM/FBS media. Irrespective, the spheroids in 24 well plates shared similar sphere morphology (Fig. 7B1 & B2). When we evaluated the expression of some selected genes (HIF-2 α , Glut1, survivin and OCT4, all the spheroids expressed these genes irrespective of the culture medium with no significant differences except for HIF-2 α and survivin

that were suggestively upregulated in both the cell lines culture in NS34 media. Similarly, these spheroids had outstanding staining pattern pointing out an intricate cellular matrix connection (Supplementary Fig. 3).

4. Discussion

The interest in the role of cancer stem cells in GBM persists due to their involvement in tumor drug resistance and relapse. GSCs are capable of both continuous self-renewal and enhanced capacity to initiate glioblastoma *in vivo* (Sharifzad et al. 2019). It is known that GSCs are a heterogeneous population present in both intratumoral perivascular and necrotic/ hypoxic niches (Ishii et al. 2016). These niches help to maintain the stemness property, regulate proliferation, self-renewal and fate and to protect the GSCs from environmental insults (Seidel et al. 2010; Fidoamore et al. 2016). An improved understanding of the molecular regulators and microenvironmental roles in tumor stem cells is crucial for designing treatment strategies aimed at the efficient eradication of this cell subpopulation. In like manner, in an effort to identify new targets for GBM diagnostics and therapeutics, studies have resulted in mounting interest in miRNAs. From our study, we were able to observe changes in the expression of miRNAs in the stem cells isolated from our parental GBM cell lines. We found miRs 34-5p, 128-3p, 181a-3p to be downregulated while miRNAs 221-3p and 17-5p to be upregulated while a comparison was made between the stem-like and their parental cells. This expression pattern was similar to the observation made when the comparison was made between the parental GBMs and a non-GBM cell line from our previous study. To support this observation, it has been reported elsewhere that miRNA profiles in stem cells resemble those seen in cancer cells (Papagiannakopoulos and Kosik 2008).

While miRNAs have been reported to contribute to CSC properties such as tumorigenicity, asymmetric cell division and chemoresistance (Osaki et al. 2015), there are limited studies reporting expression levels of miRNAs using actual glioblastoma stem cells to make an accurate comparison. Instead, most studies report the miRNA altering a gene or a pathway that plays a role in the stemness property. For example, miR-128 has been reported to be downregulated in GBM and to target the oncogene *BM11* known to regulate proliferation, self-renewal and migration of GSCs associated with enhanced self-renewal of GSCs (Godlewski et al. 2008). The miR-34 has been reported downregulated in glioblastoma and it targets key stem pathways (NOTCH1, and NOTCH2). Its forced expression induces apoptosis and inhibits invasion (Li et al. 2009a; Guessous et al. 2010) and has been shown to reduce the CSC by inducing cell differentiation of glioma cells (Guessous et al. 2010). Ectopic expression of miR-17 has been reported to facilitate the enrichment of stem-like tumor cells in GBM cells stably transfected with miR-17 and cultured in serum-free medium for two weeks. The cells showed increased capacity to form colonies and neurospheres, and expressed higher levels of CD133 (Li and Yang 2012). Among the few studies reported using actual stems, a study using global miRNA expression analysis, revealed 51 most differentially expressed miRNAs between paired GSC and non-stem cell cultures. Nine miRNAs (miR-9-3p, miR-93-3p, miR-93-5p, miR-106b-5p, miR-124-3p, miR-153-3p, miR-301a-3p, miR-345-5p, and miR-652-3p) were strongly upregulated in GSCs when a comparison was made between the GSC cluster with more pronounced GSCs features and the non-stem cell cluster (Sana et al. 2018). Similarly, a study that

evaluated the expression levels of miR-29a in GBM cells, stem cells (GSCs) and human tumors as well as normal astrocytes and normal brain by quantitative PCR found miR-29a to be downregulated in human GBM specimens, GSCs and GBM cell lines. Exogenous expression of miR-29a inhibited GSC and GBM cell growth and induced apoptosis (Yang et al. 2019). Together, these few evidences thus suggest that a stem-like state in glioblastoma can influence the expression pattern of a miRNA *in vitro*.

The effect of hypoxia on glioblastoma cell lines has been expounded in detail while less attention has been paid to the influence of hypoxia on growth and differentiation using actual cancer stem cells (Keith and Simon 2007). In this regard, our study investigated the interaction between GSCs and the hypoxia microenvironment to understand the effect of hypoxia on the *in vitro* stemness in glioblastoma. We observed that when the stem-like cells were grown under hypoxic conditions, they were larger in diameter as compared to their normoxia counterparts. Hypoxia has already been reported to enhance neurosphere formation and cell proliferation. A study that treated T4121 glioma stem and non-stem cells in hypoxia for several days noted distinct growth advantages. Cells grown in hypoxia had increased growth than the cells grown in normoxia (Heddlestone et al. 2009). We were able to observe that some neurospheres under the normoxia microenvironment had zones. This observation was made via some immunocytochemistry results where we observed that the inner zones of some spheres seemed to have higher expression of the markers under study suggesting a hypoxic situation in the middle of these spheres. It has been hypothesized that the presence of low oxygen tensions in stem cell niches offers a selective advantage that is well suited to their particular biological roles (Cipolleschi et al. 1993). Collectively, this highlights the importance of the hypoxia microenvironment in favoring the growth of the stem-like cells suggesting a cellular response sensitive to changes in the microenvironment. Most cellular adaptations under hypoxia are commonly regulated by HIFs. We observed an elevated expression of both HIF-1 α and HIF-2 α in our stem-like cells grown under normoxia and hypoxia while HIF-1 α was significantly expressed under hypoxia. It has been reported that when GBM-derived neurosphere cultures were grown in 1% oxygen, HIF1 α protein levels increased dramatically and the mRNA encoding HIF-2, was induced over 10-fold (Bar et al. 2010). It has been highlighted that HIF-2 α is preferentially expressed by CD133 + putative GSCs under both hypoxic and normoxic conditions, whereas HIF-1 α is induced under hypoxia not only in CD133 + but also in CD133- glioma cells (Li et al. 2009b). Similarly, it was observed that the knockdown of HIF-2 α in a primary GBM010 line completely blocked the upregulation of the side population signature genes, greatly suppressed the increase of CD133 levels and also abrogated the hypoxia-mediated increase in the sphere-forming capacity following hypoxia. The silencing of HIF-1 α , on the other hand, had no significant effect on CD133 levels (Seidel et al. 2010). These evidences support HIFs to play a role in cellular adaptations under hypoxia.

The miRNAs could help to modulate the survival mechanisms of the stem cells under the hypoxia microenvironment. When we compared the expression of miRNAs in the actual stem cell cultured under normoxia and hypoxia, we found miR-34-5p, 128a-3p and 181a-3p to be downregulated by a fold difference of 3.5 to 5, 2 to 5 and 2 to 4 for respectively while miR-221-3p and 17-5p to be upregulated by a fold difference of 3.5 to 4 and 2.5 to 4 respectively. There are few evidences elucidating the underlying the effect of hypoxia on miRNAs using actual GSCs. However, most evidence is based on glioblastoma

cells. HIF1 has been associated with the down-regulation of miRNAs. The miR-17-92 cluster was reported to be reduced in hypoxia-treated cells. The down-regulation of miR-17-92 by hypoxia stabilized HIF1 because this HRM is able to repress the expression of HIF1 through direct targeting (Yan et al. 2009). The miR-128 can modulate angiogenesis, functioning through suppression of *P70S6K1*, a kinase upstream of HIF-1 α and VEGF. miR-128 is decreased in gliomas which correlates with increased angiogenesis, cell proliferation, and tumor growth. Induced expression of miR-128 is able to attenuate these effects, while forced expression of *P70S6K1* can partly rescue the inhibitory function of miR-128 on cancer growth (Shi et al. 2012). Elsewhere, a study found 73 miRNAs to be differentially expressed by microarray analysis between MCF-7 spheroids cells and MCF-7-cancer stem cells as compared with their MCF-7 parental cells among which miR-210 was the most expressed (Tang et al. 2018). Upregulation of miR-210 a known hypoxia-induced miRNA, (Huang et al. 2009) indicated the presence of a hypoxic microenvironment in the MCF-7 spheroids cells and MCF-7-cancer stem cells. Elsewhere, a study that did miRNA profiling in GBM spheroid cultures grown in either 2 or 21% oxygen found miR-210 to be significantly upregulated in hypoxia in patient-derived spheroids (Rosenberg et al. 2015). These evidences show that miRNAs may represent a therapeutic target although it is not clear from the results whether this miRNA maybe related to specific cancer stem cell functions.

Besides HIF-1 α and HIF-2 α , the expression of SOX2, OCT4, GLUT-1, BCL-2 and survivin were also found to upregulated in the stem-like cells under normoxia and even further upon hypoxia induction. This observation could have been attributed to the transcription factor HIF that has been reported to regulate the expression of these genes (Covello 2006; Gordan et al. 2007; Seidel et al. 2010). Restricted oxygen conditions have been reported to expand the fraction of cells positive for a cancer stem cell marker or the side population in established cancer cell lines (Blazek et al. 2007; McCord et al. 2009). Notably, Oct4, and Sox2 are already confirmed factors that contribute to the survival and self-renewal of brain tumor stem cells (Gangemi et al. 2009; Du et al. 2009). Cancer stem cells have been shown to regulate Glut1, a transcriptional target of HIF, under hypoxia to a greater degree than non-stem cells (Li et al. 2009b). A study found Bcl-2 mRNA and its protein to be highly expressed in brain glioma stem cells when compared to their corresponding primary glioma cells. This observation was confirmed by a different study that also showed the brain cancer stem cells expressing higher levels of BCL2 protein when compared with CD133 negative cells. Therefore, as an anti-apoptotic gene, Bcl-2 assigns immortality characteristics to cells (Wang 2012; Qi et al. 2015). Upregulation of survivin has been observed in well-characterized patient-derived glioblastoma stem cell (GSC) lines on comparing the mRNA expression between GSC and non-GSC. An observation suggesting the possibility of the protein to contribute to therapy resistance in GSCs and its prognostic value in predicting postsurgical survival (Guvenc et al. 2013). Using the selected genes, we show the stem-like state and the hypoxia microenvironments to be playing a significant role in their expression that may be indirectly or directly through HIF-1 α or HIF-2 α or via the miRNAs.

The inconsistencies between the in vitro and in vivo culture models have forced the researchers to develop physiologically relevant in vitro assays that better reflect the tumor-microenvironment. The 3-D culture system can provide a simple realistic model to culture spheres that recapitulate the microenvironmental aspects creating the cell niches that can be used to study cellular interactions in

depth. When we cultured our GBM03 and T98G cell lines under our modified 3D culture system, they were all able to form spheroids by the 12th day. The spheroid morphology was not so different between the cells cultured in NS34 or DMEM/FBS. The spheroids grown in both culture mediums expressed HIF-2 α , Glut1, survivin and OCT4 by immunocytochemistry except for HIF-2 α and survivin that were suggestively upregulated in the spheroids cultured in NS34 media. Similarly, these spheroids had outstanding staining patterns highlighting an intricate cellular matrix connection. A study that compared the expression of OCT4 between 2D, neurosphere and 3D models found OCT4 to was upregulated in the 3D assays compared to the other two models (Musah-Eroje and Watson 2019). A study using MCF-7 found the cells exhibiting BCSC-like properties as shown by high expression of sox2 and oct4 when cultured as spheroid cells 3D-semisolid culture system as compared to monolayer culture (Tang et al. 2018). Both HIF-1 α and HIF-2 α have been shown to be crucially involved in the promotion of *in vitro* sphere formation, cell growth, and the survival of CD133 + glioma cells (Li et al., 2009). These evidences show that the tumour microenvironment could have been influenced reprogramming of the cell towards a stem-like phenotype (Heddeleston et al. 2009; Poli et al. 2018). Subject to further confirmation, these evidences suggest that maybe 3D culture model could be used as an alternative cheaper culture model. This is because the culture of neurospheres is time consuming as compared to the spheroid culture. Neurospheres take about four weeks to be fully formed while the spheroid culture takes less than twelve days related to the fact that true stem cells grow slower than their differentiated progeny (Kawai et al. 2016). Both culture models result in cell aggregation that can capture the hypoxia microenvironment possibly in the inner zones. Similarly, the components to making the NS34 media are expensive and to using the conventional culture media.

Conclusion

These data support the idea that oxygen tension in the stem-like cells niche functions to maintain stem cell self-renewal and an undifferentiated state. There is an aberrant miRNA expression in GSCs and these dysregulated miRNAs may be promising therapeutic targets against the GSCs. Although more preclinical works are needed to conclusively demonstrate the true nature of CSCs, discoveries regarding the function of miRNAs in CSCs will shed light on strategies for the development of specific GSC therapy. In summary, we support that hypoxia (1% oxygen) promotes expansion and maintenance of the CSC pool in GBM neurospheres. Thus, targeting the niche to disrupt the instructive and protective signals may prove an effective anti-cancer treatment. In support of the applicability of targeting the hypoxic niche, inhibition of HIF-2 may suppress tumor stem cell maintenance. Subject to further confirmation, the use of 3D culture model can be used as a cheaper alternative to capture the *in-vivo* tumor microenvironment.

Declarations

Funding

This study was supported by the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação

de Amparo à Pesquisa do Rio de Janeiro (FAPERJ) and Ary Frauzino Foundation for Cancer Research for their support.

Conflict of Interest

The authors declare no conflict of interest.

Availability of data and material

Most data generated or analysed during this study is included in the article. However, if more is needed, it can be provided upon reasonable request.

Code availability

Not applicable.

Author contributions

The article was conceptualized, written, edited and critically evaluated by each of the authors. The final preparation was done by Lucy Macharia. The work was supervised by Vivaldo Moura-Neto while the revision of the manuscript was done by Tânia Spohr. The funding acquisition was done by Vivaldo Moura-Neto. All authors read and approved the final version of the article.

Ethical statement

This study was approved by the Ethics Committee at the Center for Health Sciences at the Federal University of Rio de Janeiro (Protocol No. DAHEICB 015) and by the Brazilian Ministry of Health Ethics Committee (CONEP Protocol No. 2340)

Acknowledgements

The author is a mentee of the African Academy of Sciences (AAS) and we are thankful for their endless support and for their continued mentorship. This opportunity was as a result of the collaboration that exist between AAS and the Brazilian Academy of Sciences and for that we are grateful. The authors would also like to thank the members of the Laboratório de Biomedicina do Cérebro (LBMC) and the Associação Mahatma Gandhi for their support.

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Figures

Figure 1

Clonogenicity assay of the stem-like cells. Stem-like cells were isolated from GBM cell lines and their clonogenicity ability evaluated. The photos were taken using DMI8 Leica microscope edited using image J. Each value represents the mean $\pm$ SD of three independent runs. Scale bar = 200 μ m. The stem-like cells demonstrated high clonogenicity ability and a gradual size increment of the clones with increasing time.

Figure 2

Expression of miRNAs in GBM stem-like cells by qRT-PCR. The GBM stem-like cell lines were evaluated for the expression of miRNAs by qRT-PCR. The relative fold change difference was calculated using $2^{-\Delta\Delta Cq}$ method where RNU6 was used as the normalizer. Each value represents the mean $\pm$ SD of three independent runs where, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the GBM cells. miR-34, 181, and 128 similarly maintained their downregulated expression while miR-17 and 221 maintained their upregulated expression in the stem like population like the GBM cells.

Figure 3

Expression of HIF-1α and HIF-2α after the hypoxia acquisition. The stem-like cell lines were cultured under normoxia (N) and in hypoxia (H) for 72hours and the expression of HIF-1/2α evaluated by immunofluorescence A. and qRT-PCR B where the relative fold change difference was calculated using 2- $\Delta\Delta CT$ method where ACTIN was used as the normalizer. Each value represents the mean $\pm$ SD of three independent experiments where, *p < 0.05, **p < 0.01, ***p < 0.001. Scale bar = 50 μm.

Figure 4

Effect of hypoxia on the expression of miRNAs in the stem-like cells. The stem-like cells were cultured under normoxia and hypoxia conditions. The differential expression of miRNAs was evaluated by qRT-PCR and the relative fold change difference calculated using $2^{-\Delta\Delta Cq}$ method where RNU6 was used as the normalizer. Each value represents the mean $\pm$ SD of three independent runs where, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; compared to the stem-like cells under normoxia.

Figure 5

Enhanced expression of transcription factors under hypoxia microenvironment. The stem-like cells were cultured under normoxia (N) and in hypoxia (H) for 72hours and stained for SOX2 and OCT4 (1:400) by immunofluorescence. Each value represents the mean $\pm$ SD of three independent experiments where *p < 0.05, **p < 0.01, ***p < 0.001. Scale bar = 50 μ m. The stem-like cells expressed SOX2 and OCT4 but the expression under normoxia was strongest in the inner zones of the neurospheres.

Figure 6

Increased Apoptotic escape and metabolic reprogramming under hypoxia. The stem-like cells were cultured under normoxia (N) and in hypoxia (H) for 72hours and stained for survivin, Bcl2 and Glut1 by immunofluorescence. The Images were taken with DMI8 Leica microscope and prepared and quantified using ImageJ. Each value represents the mean $\pm$ SD of three independent experiments, where *p < 0.05, **p < 0.01, ***p < 0.001. Scale bar = 50 μm.

Figure 7

Morphological changes of the glioblastoma spheroids. The GBM 03 and T98G parental cells were cultured in 24 and 96 well plates in either NS34 or DMEM/FBS in 3D modified culture models for a period of 12 days. The photos were taken using DMI8 Leica microscope and edited using image J. Scale bar = 50 μ m. Each experiment was done at least 3 times.

Supplementary Files

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

- [Supplementaryfigure1Macharia2021b.tif](#)
- [Supplementaryfigure2Macharia2021b.tif](#)
- [Supplementaryfigure3Macharia2021b.tif](#)