Expression of pluripotency-related genes in human glioblastoma.

Álvaro Fabrício Lopes Rios¹ – lab.biomol.cirurgia@fmrp.usp.br
Daniela Pretti da Cunha Tirapelli² – daniela@fmrp.usp.br
Mucio Luiz de Assis Cirino² – mucioassis@usp.br
Andressa Romualdo Rodrigues³ – dreromualdo@gmail.com
Ester S. Ramos⁴ - esramos@fmrp.usp.br
Carlos Gilberto Carlotti Jr² – carlotti@fmrp.usp.br

¹Laboratory of Biotechnology, Center for Biosciences and Biotechnology, North Fluminense State University, Campos dos Goytacazes, Rio de Janeiro, Brazil.

²Department of Surgery and Anatomy, Ribeirão Preto Faculty of Medicine, University of São Paulo, Ribeirão Preto, São Paulo, Brazil.

³ Laboratory of Morphofunctional and Integrated Practices, Franca Medical School, University of Franca, Franca, São Paulo, Brazil.

⁴Department of Genetics, Ribeirão Preto Faculty of Medicine, University of São Paulo, Ribeirão Preto, São Paulo, Brazil.

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Corresponding author: lab.biomol.cirurgia@fmrp.usp.br
Álvaro Fabrício Lopes Rios
Ribeirão Preto Medical School, University of São Paulo
Av Bandeirantes, 3900. Ribeirão Preto, SP, Brasil 14049-900
Phone: +55 16 3315 3305
E-mail: lab.biomol.cirurgia@fmrp.usp.br
The corresponding author is the Guarantor of Submission.

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Conflicts of Interest
The authors declare no conflicts of interest.

Authorship
- Álvaro Fabrício Lopes Rios - was involved in the acquisition and analysis of the data, manuscript writing.
- Daniela Pretti da Cunha Tirapelli - was involved in the conception and design intellectual and scientific content, supervision of all phases of the study, and critical revision.
- Mucio Luiz de Assis Cirino – was involved in manuscript drafting and critical review of intellectual content.
- Andressa Romualdo Rodrigues – was involved in manuscript drafting and critical review of intellectual content.

- Ester S. Ramos - was involved in the conception and design and scientific content of the study, supervision of all phases, and critical revision.

- Carlos Gilberto Carlotti Jr – was involved in the conception and design and scientific content of the study, supervision of all phases, and critical revision.
Abstract

Background: Cancer is a group of heterogeneous diseases characterized by several disruptions of the genetic and epigenetic components of cell biology. Some types of cancer have been shown to be constituted by a mosaic of cells with variable differentiation states, with more aggressive tumors being more undifferentiated. In most cases, undifferentiated tumor cells express associated embryonic markers such as the OCT4, NANOG, SOX2 and CARM1 genes. The ectopic or reminiscent expression of some master regulator genes of pluripotency has been indicated as the cause of the poorly differentiated state of tumors, and based on the evidence of some reports, can be used as a possible therapeutic target. Considering this information, a more detailed investigation of the expression of pluripotency-associated genes is necessary to evaluate the roles of these genes in the etiology of some tumors and their use targets of therapy. Methods: The expression of four pluripotency-related genes was investigated (OCT4, NANOG, SOX2 and CARM1) in the most malignant primary human brain tumor, glioblastoma (GBM). Results and Conclusion: The results demonstrated a signature of OCT4/SOX2/CARM1 genes and a significant increase of CARM1 expression in GBM cases.

Key words: cancer, pluripotency, stem cell, gene expression.
Key points

- Study of human glioblastoma samples.
- Identification of a signature of pluripotency-related genes that may help to elucidate tumorigenesis and treatment of GBM.

Importance of the study

This study describes some characteristics of glioblastoma, a tumor type is highly aggressive usually fatal. Identifying a pluripotency-related gene signature is a very important step for medical science.
Introduction

Glioblastoma (GBM) or astrocytoma grade IV according to the World Health Organization (WHO) classification is the most aggressive and common of all primary brain tumors, accounting for approximately 40% of all primary and 78% of all malignant central nervous system tumors\textsuperscript{1,2}. Mutations (TP53, p16\textit{INK4a}, p14\textit{ARF}, and PTEN), amplifications (EGFR and CDK4/6) and loss of heterozygosity (LOH) of several chromosomes (1p, 6q, 9p, 10p, 10q, 13q, 14q, 15q, 17p, 18q, 19q, 22q, and Y) are among the major genetic alterations found in GBMs\textsuperscript{3-5}.

The epigenetic changes identified in GBMs include the silencing of genes involved in many biological processes, such as cell cycle regulation, DNA repair and apoptosis. Among these genes are MGMT, MLH1, p16\textit{CDKN2A}, RASSF1A, PTEN, TP53, p14\textit{ARF}, AR, WT1, CDH1, p15\textit{CDKN2B}, MT1A and RB\textit{1}\textsuperscript{6,7}. The stem cell theory of cancer proposes that cancers arise from stem cells that are present in all tissues\textsuperscript{8-10}.

According to this theory, cancerous tissues are similar to normal tissues, being composed of stem cells, transit-amplifying cells, and terminally differentiated cells\textsuperscript{11}. In recent years, various studies have reported the presence of stem cells in brain tumors, referred to as cancer stem cells (CSCs)\textsuperscript{7-13}. CSCs are multipotent, meaning they have the property of self-renewal and are believed to be responsible for initiating and maintaining tumors, recurrence, and therapy resistance\textsuperscript{13,14}.

The presence of stem cell-like phenotypes implies the presence of molecular networks governing them. Several recent studies have detected the expression of pluripotency-associated genes such as OCT4, SOX2 and NANOG in human tumors\textsuperscript{15-17}. Together, these genes are responsible for the regulation of several
hundred genes involved in establishing pluripotency and stem cell differentiation *in vitro*\(^{18}\).

OCT4 and NANOG are homeobox transcription factors and SOX2 is a member of the Sox (SRY-related HMG box) gene family, which encodes transcription factors with a single HMG DNA binding domain\(^{19}\).

These three factors form a core regulatory network that coordinately determines embryonic stem cell (ESC) self-renewal and differentiation. Up or downregulation of OCT4 and NANOG induced *in vitro* may allow the entry of undifferentiated cells into differentiation pathways. In human stem cells, the reduction of OCT4 expression promotes upregulation of mesoderm and endoderm markers, whereas increased levels of OCT4 promote upregulation of endoderm markers\(^{20}\). Another important gene recently associated with the pluripotency phenotype is the coactivator-associated arginine methyltransferase 1 - CARM1 gene, also called PRMT4\(^{21}\).

Torres-Padilla et al. (2007) demonstrated that CARM1 is required for self-renewal and pluripotency during early embryonic development. They also demonstrated that CARM1 is associated with the Oct4 and Sox2 promoters, which display detectable levels of H3 R17 and R26 methylation. During early development, Carm1 seems to exert a positive influence on the expression levels of Nanog and Sox2 genes\(^{22}\). CARM1 is also associated with the activation function of the tumor suppressor gene TP53, acting directly with its protein sequence\(^{23}\). CARM1 downregulation induced by RNAi causes loss of the pluripotency phenotype in cells after knockdown\(^{24}\).

These four genes are key components of the complex circuit involved in stem cells self-renewal and pluripotency, which can also act in undifferentiated tumors\(^{19,25}\).
Additional supporting evidence of the action of stem cell-related genes in tumors has been presented in many studies, which have identified a stem cell-like signature in poorly differentiated tumors, including glioblastoma. Reacquired stem cell-like characteristics can involve drug resistance, self-renewal, and embryonic-like gene expression signatures\textsuperscript{14}.

A regulatory relationship between pluripotency cell markers and drug resistance was reported by Chambers et al. (2004), who showed that Oct4, when overexpressed by a 3-fold factor, binds to the promoter region of ABCB1, ABCG2, and ABCC1 in MDR K562-Lucena cells. In contrast to all the undesired properties of this embryonic molecular network, some favorable factors can be present. These factors act on thousands of developmental genes, and experimental disruption of some of these factors causes differentiation of disrupted cells and loss of embryonic features\textsuperscript{26,27}.

Understanding the common molecular networks between cancer stem cells and their normal counterparts is vital to discover the real importance of these cells in tumorigenesis processes. Advances in knowledge may help in the development of new therapeutic approaches aiming to stop the development of CSC. The aim of this study was to investigate the expression of four pluripotency-related genes (OCT4, NANOG, SOX2 and CARM1) in human glioblastoma (GBM).

**Patients and tissue samples**

For this study two groups of samples were investigated. One was composed glioblastoma samples from 22 patients (16 men and 6 women) who underwent tumor resection at the Clinical Hospital of Ribeirão Preto Faculty of Medicine, University of São Paulo. Tumor grade was determined according to the WHO criteria\textsuperscript{2,30}. The second group consisted of 10 samples of nonneoplastic white matter obtained from
patients (4 men and 6 women) who underwent cortico-amygdalo-hippocampectomy for epilepsy treatment at the same hospital. The study was approved by the Ethics Committee of the Faculty of Medicine and informed consent was obtained from each patient. All tissue samples were microdissected for exclusion of tissue areas presenting necrosis or not matching GBM diagnostics prior to RNA extraction.

Primer design

To avoid sequence amplification of the expressed pseudogenes of NANOG and OCT4, as criticized by some authors, we performed a careful design of the primer pairs used in this study\textsuperscript{31,32}. The primers used for NANOG and OCT4 analysis were selected by aligning the transcribed sequences of these genes and their respective pseudogenes using the Multialin software\textsuperscript{33}. The primer sequences of these two genes were selected due to lack of identity with the pseudogenes and subsequent analysis with the Gene Runner v.3.05 software (Hastings Software Inc.).

The other primer sequences used were designed using GeneRunner (CARM1 gene) or selected from the work of Valente et al. (2009) (TBP and HPRT1 genes)\textsuperscript{34}. The 5’ to 3’ sequences of all forward (F) and reverse (R) primers used were: OCT4A-F TCCCTTCGCAAGCCCTCAT and OCT4A-R CACCACCTGGAGGGGGCG; NANOG-F TTATAAATCTAGAGACTCCAG and NANOG-F GAGAAATAGGACCTCCAGAAG; CARM1-F CTACCTCCACGCAAGAAG and CARM1-R GGTTGAACGTCCATGTAGA; HPRT1-F TGAGGATTTGAAAGGTGT and HPRT1-R GACGACACAGAAGGCTACAA; TBP-F GAGCTGTGATGTAAGTTTCC and TBP-R TCTGGGTTTATCATTCTGTAG; β-ACTIN-F CTGCTTCCAGCTCCTCC and β-
ACTIN-R AGTTTCGTGGATGCCACAGG; GAPDH-F GTCGCCAGCGGAGCCACA and GAPDH-R GGGTGGAATCATATTGGGAACA.

**Total RNA extraction and cDNA synthesis**

Total cellular RNA was extracted using Trizol® reagent (Invitrogen, Carlsbad, CA, USA) and subsequently submitted to DNase I treatment to eliminate any possible contamination with genomic DNA (gDNA). Samples of 500 ng of total RNA were digested using 1U of DNase I (Invitrogen) at room temperature for 15 minutes and inactivated by the addition of 1 µl of EDTA (25 mM) and incubated to 65 °C for 5 minutes at a final volume of 10 µl. The DNase I treated RNA was reverse transcribed to single-stranded cDNA using a High-Capacity Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol.

**Qualitative and quantitative PCR (qPCR)**

The cDNA samples produced were diluted 10-fold and tested for possible gDNA contamination by amplification of a fragment of the human β-actin gene amplified with a primer pair anchored in two different exons with an intervening intron. After finding no gDNA contamination, the samples were used for real-time polymerase chain reaction (PCR) (unpublished data). The transcript levels of each studied gene were evaluated using an ABI 7500 system (Applied Biosystems, Foster City, CA, USA).

For relative quantification (RQ) of gene expression, standard curves were constructed for each gene by considering at least 3 points in triplicate of the 10-fold serial dilution of cDNA in water, starting from a 1:10 volume of undiluted cDNA transcribed from 500 ng of total RNA. To normalize differences in the amount of total
cDNA added to each reaction, TBP and HPRT gene expression were used as endogenous control. As a calibrator sample (reference sample for relative quantification), the NTERA2 cell line was used. All reactions were performed in duplicate, and all procedures were carried out at 4 °C.

**Statistics**

GBMs and control samples were compared using the Mann-Whitney test, except for OCT4A analysis, in which the data were submitted to Student’s t-test. All tests were two-tailed and statistical significance was considered to be \( p < 0.05 \).

**Results**

**Primer efficiency of OCT4, NANOG, SOX2, and CARM1**

The primer efficiency of real-time PCR (\(10^{-1}/\text{slope} -1\)) of the set of primer pairs used for this study ranged from 95 to 100%.

**Analysis of the pluripotency-related genes expression in glioblastomas.**

The quantitative results from the quantitative RT-PCR also demonstrated the presence of OCT4 transcripts in two control samples (Fig. 2). The relative quantification (RQ) of OCT4 transcripts in GBM versus control samples demonstrated a 14.77-fold increase in GBMs, besides the large difference between the median of the two groups, which was statistically significant according to Student’s t-test (\( P = 0.3561; P < 0.05 \)). Despite no differences of expression being found, SOX2 was present in 91.66% of the analyzed samples and co-detected with OCT4 in 70.83% of them (Fig. 3). Analysis of the CARM1 expression comparing the RQ medians showed a 2.84-fold increase in the GBM samples versus the control. The result of the analysis of CARM1 expression demonstrated a significant
difference between the groups analyzed (Mann-Whitney, test P = 0.0201, P < 0.05) (Fig 2). NANOG expression was not detected in either the GBMs or the control samples. The detection of NANOG sequences in tumors has been attributed to its pseudogene number 8, which is not expressed in NTERA2 cells (the calibrator sample used in this study), which were positive for NANOG expression in our assays (unpublished data).

Discussion

The interpretation of the data of the expression of pluripotency-related genes in cancer has been brought into question because of the large number of processed pseudogenes or even duplicated sequences present in the case of these genes. Of the 22 GBM samples analyzed, 19 (86.36%) displayed OCT4 expression. Our results diverge slightly from the findings of Du et al. (2009), who found 100% (19/19) of GBM samples positive for OCT4. However, the primers used by those authors did not present a significant difference between the OCT4 and their pseudogenes (our unpublished data).

The expression of OCT4 pseudogenes has been detected in cancer cell lines as well as cancer tissues. An additional point that needs to be verified is the presence of two isoforms of OCT4 genes. Cauffman et al. (2006) reported in detail the two main splice variants (OCT4A and OCT4B). Functionally, the two human OCT4 isoforms showed different expression profiles, with only OCT4A being expressed in hESC.

These isoforms also differ in their ability to confer self-renewal. I contrast to OCT4A, OCT4B does not have this property or capacity to bind to the OCT4 consensus binding sequence. The human OCT4A isoform is clearly located in the
nucleus, whereas OCT4B is mainly expressed in the cytoplasmic compartment of the cell\textsuperscript{37}. The study of the specific OCT4A transcript was focused to better investigate whether a real molecular embryonic signature was present in GBMs. Together with SOX2 factors, OCT4 acts on the regulation of thousands of genes by dimer-formation and even co-occupancy of regulatory regions in the genome\textsuperscript{38-40}. Using data generated from ChIP-seq in GBM cells, Fang et al. (2011) found 2323 binding sites of OCT4 mapped within -2kb of the TSSs in glioblastoma cancer cells\textsuperscript{41}. By enrichment analysis using Gene Ontology (GO), these authors found that the genes bound by OCT4 are involved in some important biological processes, including gene expression, translation, mRNA processing, wound healing, and apoptosis\textsuperscript{41}. The number of genes bound by OCT4 and the biological processes associated with them provide important evidence of OCT4’s role in GBM biology. In addition, as further evidence of the functional role of OCT4 in GBM, the inhibition of OCT4 and AKT potently suppresses the propagation of glioblastoma cell lines U87 and U251 in vitro\textsuperscript{42}.

The co-expression of OCT4 and SOX2 in the context of in vivo GBM cells is also important to clarify the role of these genes in important biological processes associated with the development of these brain tumors. Both OCT4 and SOX2 expression are detected in glioblastoma stem cells (GSCs), which survive chemical treatment. These surviving GSCs have the capacity to recapitulate the tumor\textsuperscript{43}.

SOX2 plays a critical role in the carcinogenesis and maintenance of GBM stem cells, associated with resistance to chemotherapy and radiotherapy\textsuperscript{44}. The same authors reported that increased SOX2 expression enhanced stem cell potency in GBM cell lines, while downregulation of this gene was associated with abrogated ability of tumor initiation and drug resistance of CD133+ GBM cells\textsuperscript{44}. 


The results of biological function and overexpression of CARM1 presented here may indicate a new therapeutic target to attack human glioblastoma.

CARM1, which methylates arginine residues at histone 3, is targeted to promoter sequences of the Oct4, Sox2, and Nanog genes, shown to be intrinsically involved in pluripotency\textsuperscript{24}. Downregulation of CARM1 in murine ESCs causes the cell to enter the differentiation pathways. Reduction of the transcribed level of CARM1 by RNAi, and consequently their codified protein, causes the reduction in transcriptional levels of Oct4, Sox2 and Nanog, and an increase in the expression of differentiation-associated genes\textsuperscript{24}.

CARM1 is associated with the regulation of OCT4, SOX2, and NANOG, as indicated by the downregulation of these three genes in human embryonic stem cells submitted to knockdown of CARM1\textsuperscript{45}. Conversely, overexpression of CARM1 increases cells’ resistance to differentiation.

Similar to our results, increased CARM1 expression was found in glioblastoma samples by Kappadakunnel et al. (2010)\textsuperscript{46}. They reported increased expression of the CARM1 gene in GBM tumors in contact with the subventricular zone (SVZ), and associated this altered expression pattern with poorer patient survival.

Increased CARM1 expression and/or activity have previously been reported in many different cancer types, including prostate, breast, colorectal, lung, and liver cancer\textsuperscript{47-49}. In solid tumors, CARM1 acts as a cofactor for the transcription factor NF-κB, p53, steroid hormone receptors and, it is functionally related to cancer cell proliferation, metastasis and poor survival outcomes\textsuperscript{50}.

The expression of NANOG has also been detected in many cancers, but similar to OCT4, it has many pseudogenes anchored at different chromosomal
positions\textsuperscript{45}. For the NANOG primer pair, the results also demonstrated primer specificity for amplification of this gene. In addition to the bioinformatics data (Electronic Supplementary Material Fig.1), the RT-PCR results demonstrated the amplification of the NANOG fragment from NTERA2 cDNA (Fig. 1), which expresses NANOG but not NANOFP8\textsuperscript{16}. Early pluripotency signatures were reported by Kashyap et al. (2009) for genes regulated by OCT4, SOX2 and NANOG. In the present work, two of the three master regulators of pluripotency expressed in human glioblastoma were found, as well as increased expression of CARM1, which is involved in the regulation of both factors\textsuperscript{43}.

Knockdown induced by shRNAs against CARM1 mRNA in glioblastoma (LN229) and neuroblastoma [BE(2)-C] cell lines inhibited the proliferation in both cell types, demonstrating the functional impact of this gene in both brain tumors\textsuperscript{51}. These authors also found that CARM1 downregulation was associated with decreasing mRNA or protein levels of LDHA, GLUT1 and ASCT2 genes. The results of Wang et al. (2020) suggest that CARM1 may be involved in the regulation of glycolysis and glutaminolysis in glioblastoma and neuroblastoma. Since both these metabolic pathways promote cell proliferation in multiple cancers\textsuperscript{52}, they could contribute to molecular mechanisms associated with the effects of CARM1 in the cell proliferation of these tumors.

The great number of genes whose expression is affected by these genes and the results of functional studies manipulating their expression suggest that these may be involved in the stem cell features present in GBMs. The biological significance of some of these factors for pluripotency can be observed from studies of the production of induced pluripotent stem (iPS) cells. The simultaneous presence
of OCT4 and SOX2 was shown to be essential to achieve success in iPS cell production, and sometimes the use of only these two factors was sufficient\textsuperscript{19}.

The central position occupied by OCT4, SOX2 and CARM1 in the control of pluripotency in undifferentiated cells may point the way to valuable opportunities to disrupt cell phenotypes associated with the survival and proliferation of cancer stem cells. Loss of these factors could disrupt important characteristics present in undifferentiated cells, such as self-renewal, drug resistance, and blocking of differentiation pathways. In this context, the expression of these three factors may indicate specific and effective targets for therapeutic intervention. Early knockdown experiments in cancer cells demonstrated that disruption of the gene expression of OCT4 caused differentiation in embryo carcinoma\textsuperscript{27}. Knockdown of SOX2 stopped proliferation and induced the loss of tumorigenicity in immunodeficient mice\textsuperscript{15}.

In addition to the aforementioned data, it is important to note that many of the molecular signatures of these genes were found in studies using GBM cell lines from commercial sources or cell cultures derived from GBM tumors. In our study, we analyzed these molecular signatures in samples derived from tumor tissues obtained directly from patients, without any manipulation by \textit{in vitro} culture protocols, reinforcing the presence of a pluripotent molecular signature in GBM cells \textit{in vivo}. So, we believe that the same functional effects derived from the expression or the knockdown of these genes observed in \textit{in vitro} culture studies may occur \textit{in vivo} as well. The gene expression patterns found in our study in GBM samples indicate the occurrence of a stem cell-like molecular signature and identify these genes as possible targets in future therapeutic protocols.
Conclusions

Embryonic stem cell gene expression signatures for OCT4A, SOX2, and CARM1 genes in GBM samples were found, and given their well-documented influences on an array of downstream targets, we suggest that the expression of these factors can be involved in the embryonic-like features present in these tumors, as previously documented by other authors. We also suggest that the expression of OCT4A, in two non-neoplastic samples, could represent a possible stem cell niche with at least one embryonic feature in adult brain tissue. As far as we know, this is the first report of the increased gene expression profile of CARM1 in addition to a stem cell-like signature involving SOX2 and OCT4 expression in GBM.
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Fig. 1 RT-PCR analysis of specific amplification by specific primer pairs of OCT4A and NANOG transcripts in: (1) NTERA2, an undifferentiated cell expressing NANOG and OCT4A but not NANOGP8; (2) peripheral blood cell cDNA (differentiated cell pool), negative for OCT4A [11, 25]. L50 – ladder 50 base pairs.

Fig. 2 Graphic representation of differences of fold expression between GBM and control samples derived from quantitative RT-PCR of the genes: (a) OCT4 (P = 0.3561, P < 0.05); and (c) CARM1 (P = 0.0201, P < 0.05).

Fig. 3 Percentage of GBM samples presenting one of the respective molecular signatures: OCT4-SOX2- CARM1 (70.83%), OCT4-CARM1(8.33%) and SOX2-CARM1 (20.83%).
Figure 1

- OCT4
- NANO
- GAPDH
Figure 2

**OCT4A**

- GBM
- Control

**SOX2**

- GBM
- Control

**CARM1**

- GBM
- Control
Figure 3

The diagram shows the percentage distribution of different combinations:

- OCT4-SOX2-CARM1: 70.83%
- OCT4-CARM1: 8.33%
- SOX2-CARM1: 20.83%

The percentage values are indicated above each bar.