Review

Adjusting the Clock: the Importance of Circadian Rhythms in the Development of Glioblastomas and Its Intervention as a Therapeutic Strategy

Paula M. Wagner a,1,2; César G. Prucca a,1,2; Beatriz L. Caputto 1,2 and Mario E. Guido *1,2

1 CIQUIBIC-CONICET, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, 5000 Córdoba, Argentina.
2 Departamento de Química Biológica Ranwel Caputto, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, 5000 Córdoba, Argentina.
* Equal contribution

Abstract: Gliomas are solid tumors of the Central Nervous System (CNS) that originated from different glial cells. The World Health Organization (WHO) classified these tumors into four groups (I-IV) with increasing malignancy. Glioblastoma (GBM) is the most common and aggressive type of brain tumor classified as a grade IV. GBM are resistant to conventional therapies with poor prognosis after diagnosis even when the Stupp protocol that combines surgery and radiochemotherapy is applied. Nowadays, few novel therapeutic strategies have been used to improve GBM treatment, looking for higher efficiency and lower side effects, but with relatively modest results. The circadian timing system temporally organizes the physiology and behavior of most organisms and daily regulates several cellular processes in organs, tissues, and even in individual cells, including tumor cells. The potentiality of the function of the circadian clock on cancer cells modulation as a new target for novel treatments with a chronobiological basis offers a different challenge that needs to be considered in further detail. The present review will discuss state of the art regarding GBM biology, the role of the circadian clock in tumor progression, and new chrono-chemotherapeutic strategies applied for GBM treatment.

Keywords: CIRCADIAN RHYTHMS, GLIOBLASTOMA, TREATMENT

Introduction

Gliomas are solid tumors of the Central Nervous System (CNS) that originated from different glial cells that share histological features with astrocytes, oligodendrocytes, and ependymal cells [1,2]. Based on morphological and histochemical features, the World Health Organization (WHO) classified CNS tumors into four groups (I-IV) with increasing malignancy. Glioblastoma (GBM) is classified as grade IV glioma [3] with a mean age of 64 years at diagnosis and an incidence of 3.19 cases per 100,000 population [4]. GBM is the most common and aggressive type of brain tumor, representing 45.2% of all malignant CNS tumors and 80% of all primary malignant CNS tumors [5]. These malignancies are originated mainly from primary gliomas (90%), while a small proportion (10%) arise as secondary gliomas deriving from lower-grade tumors [2], even when both of them can be histologically indistinguishable [6]. Originally, GBM was thought to be rising solely from glial cells; however, recent evidence suggests three different cell types as the origin of GBM: neural stem cells (NSCs), NSC-derived astrocytes, and oligodendrocyte pre-
cursor cells (OPCs). The cellular origin is a significant determinant for the molecular subtype classification and may contribute to tumor development [7]. A well-known feature of GBM is its high cellular heterogeneity characterized by mutant cells with several morphologies, different levels of aneuploidy, and differential expression of specific cell markers. In addition, particular transcriptional programs controlling cellular processes such as hypoxia, cell cycle, and immune signaling have been observed in individual cells [8]. Among the histological features of these highly infiltrative tumors, regions of necrosis, microvascular proliferation, abundant mitoses, and pleomorphic cells are the most important and representative [1].

For patients with newly diagnosed GBM, the surgical approach is the mainstay of treatment. However, complete tumor resection is often not possible due principally to the lack of clear tumor boundaries, the infiltrative nature of this type of tumors, and the risk of aggressive resection leading to postoperative neurologic deficits [9–11]. Additionally, GBM is a highly diffusive, invasive, and vascularized tumor. All these mentioned features make GBM not fully curable with surgical intervention alone. Therefore, Temozolomide (TMZ), an oral imidazotetrazine alkylating agent which induces methylation of DNA, combined with radiotherapy, has become the established standard of care treatment after surgical resection [12]. However, these high-speed growth tumors are resistant to conventional therapies and are associated with poor prognosis, showing a median overall survival (OS) of 12–15 months for patients with newly diagnosed GBM [1,12]. Intratumoral heterogeneity, defined as the presence of multiple different cell subpopulations within a single tumor from one patient [13], is believed to contribute to the resistance and recurrence rate observed in GBM. Several mechanisms that cooperate to this heterogeneity have been proposed, such as the presence of cancer stem cells (CSCs) in the tumor with varying degrees of stemness and their ability to self-renew and differentiate into different types of tumor cells; heterogeneity further potentiated due to the genetic instability of the cells that leads to the generation of different subclones inside the tumor, selected by their resistance to treatment [14,15]. Besides these hypotheses, several factors could influence tumor heterogeneity, including the presence of epigenetic alterations and interactions among tumor cells and between them with the tumor microenvironment [15]. Also, intratumor heterogeneity is spatially influenced since the evidence shows that biopsies taken from the tumor core and interface zones present higher levels of genomic alterations as compared to biopsies from the peripheral brain zone, suggesting that the changes observed in the gene expression profile are dependent upon tumor area [16].

Despite the significant progress in the research of novel therapies to treat GBM reported in the last years, fewer than 5% of the patients survive for 5 years after diagnosis [17]. Therefore, innovative approaches urgently need to be investigated to increase the quality of life and survival of patients. Over the last few years, some authors proposed optimizing anticancer drug delivery by timing it to the daily rhythms of the host [18,19]. Circadian clocks temporally organize the physiology and behavior of most organisms, including humans, by generating daily rhythms in several physiological processes, including sleep/wake cycles, behavior, locomotor activity, body temperature cycles, cardiovascular and digestive processes, endocrine systems, and metabolic and immune functions with an intrinsic 24-hours period oscillation [20]. Chronotherapy has emerged as a new concept that proposes the differential administration of drugs throughout the 24-hours daytime to improve drug efficiency and reduce cancer toxicity and side effects of the treatment. This strategy has been applied only recently to treat CNS tumors because it was shown a differential response to the proteasome inhibitor Bortezomib in a murine model [21] and that TMZ administration in human and murine GBM cells in culture is dependent on clock gene expression [22]. In this review, we analyze and discuss the current literature regarding GBM biology, the role of the circadian clock in tumor progression, specifically on GBM, and the potential of chronotherapy as an original approach to improve the treatment for this type of tumor.
GBM general hallmarks

Gliomagenesis, as a multi-component process that promotes the development of gliomas, involves amplification and deletion or mutation of several genes, including the epidermal growth factor receptor (EGFR), tumor protein 53 (TP53), phosphate and tensin homolog (PTEN), and isocitrate dehydrogenase (IDH), among others [23]. These genes regulate distinct pathways known to be part of the core drivers of gliomagenesis, leading to aberrant signaling in proliferation, cell cycle regulation, senescence, and apoptosis [24,25].

One of the most studied hallmarks of human GBM is the amplification and genetic rearrangement of the gene that encodes for the tyrosine kinase receptor known as EGFR. This pathway can be activated either through overexpression of the receptor, amplification of the EGFR locus, and/or mutations in the receptor [26]. The most common and described mutation in GBM is the EGFRvIII, which corresponds to the loss of exons 2-7, resulting in a truncated extracellular domain with ligand-independent constitutive activity and consequently excessive cell proliferation. This mutation is associated with a bad prognosis and has not been observed in healthy tissues and secondary GBM subtypes [27]. Interestingly, GBM cells express either EGFR or EGFRvIII, although co-expression of both variants has also been reported in a small population of cells [2]. The TP53 gene encodes for a tumor suppressor protein that participates in cell cycle control, DNA damage response, cell death, and differentiation. Its mutation incidence is low in primary tumors (about 30%); however, 90% of secondary GBM present mutations in this gene. Indeed, it has been proposed that TP53 mutation is an early event in secondary GBM [6] and is correlated with GBM progression by driving the activation of the mevalonate pathway since p53-mutant cells have shown an elevated activity of this pathway as compared to wild type cells [28]. Other alterations related to this pathway include murine double minute 2 (MDM2) and MDM4 amplification, and CDKN2A-p14ARF deletion [24]. As MDM2 is a negative regulator of the TP53 gene, using inhibitors of MDM2 has shown promising results in GBM treatment [29]. PTEN gene is another most commonly mutated tumor suppressor gene observed in most cancers, similar to TP53 [30]. It has a crucial role in inhibiting cell proliferation and regulating the migration and invasion of cells. PTEN is frequently inactivated in GBM, either by losing heterozygosity (LOH) of chromosome 10 or by mutation-induced constitutive activation of PI3K. The LOH of chromosome 10 is observed in almost 70% of GBM samples, predominantly in the primary subtype [23]. Amplification of platelet-derived growth factor receptor (PDGFR) is another genetic alteration observed in GBM tumors [31]. Lastly, IDH mutations are considered the most reliable indicator to differentiate primary from secondary GBM [32–36], being primary GBM those that typically lack IDH mutations [37]. IDH gene encodes for isocitrate dehydrogenase, an enzyme that catalyzes the oxidative decarboxylation of isocitrate to 2-oxoglutarate within the Krebs cycle, whereas IDH mutants catalyze the production of the oncometabolite 2-hydroxyglutarate (2-HG). Importantly, patients treated with TMZ are associated with a favorable prognosis when they present IDH mutations since the synthesis of 2-HG interferes in the activation of DNA demethylation enzymes, yielding a hypermethylation status in tumor cells [38].

The O6-methylguanine-DNA methyltransferase (O6-MGMT) gene encodes for an enzyme that removes the methyl group from the guanine (position O6). The expression level of this protein is relevant to the treatment outcome when using TMZ since its expression is associated with a poor response. Consequently, the methylation level of its promoter is associated with a better response to TMZ treatment [23].

GBM classification
The WHO classified brain tumors into four groups (I-IV) of growing malignancy based on the morphological features of the tumor and its cell of origin [39]. Grade I and II include tumors with low proliferation potential, whereas grade III and IV tumors are high-grade gliomas characterized by high proliferation rates and aggressiveness [40]. GBM is classified as a grade IV high-speed growth tumor showing diffuse boundaries and is usually associated with a poor prognosis [6]. GBM is also divided into primary and secondary tumors, being the primary GBM commonly diagnosed in the elderly without prior disease. Several alterations have been reported in primary GBM including LOH at 10q (70% of cases) [27,32,41] and 10p (50-70%) [27,42], amplification or mutation of EGFR (~35-45%) [27,32,41–43] mutation in TP53 (27-30%) [27,43], deletion of cyclin-dependent kinase inhibitor 2A/B (CDKN2A/B) (31%) [44], mutation or deletion of PTEN (25%) [27], promoter methylation of O6-MGMT (42%) [45], promoter mutation of telomerase reverse transcriptase (TERT) (72%) [41,43], mutation in glioma-associated oncogene homolog 1 (GLI1) (5–22%), deletion or mutation in phosphatidylinositol-4,5-bisphosphate 3-kinase A (PIK3CA) (~1%), MDM2 (7-12%) [41,42,44] and neurofibromatosis type1 (NF1) (11%), amplification of PDGFR (7%), and mutation in IDH1/2 (5%) [44].

On the other hand, secondary GBM develops from a low-grade glioma or an anaplastic astrocytoma that affects younger persons and shows a better prognosis after diagnosis. These tumors are much less common showing genetic alterations that include mutation in IDH1/2 (73-85%) [46], TP53 (65-81%), ATRX (~65-71%) [27,43], and PTEN (<5%) [27] genes, promoter methylation of MGMT (79%) [45], loss of chromosome 19q (~50%) and 10q (63%) [27], p16INK4a deletion (19%) and EGFR amplification (8%) [32]. Mutant IDH1 is considered a metabolic marker of secondary GBM because of its ubiquitous expression in lower-grade gliomas that eventually progress to GBM. Besides the differences described above regarding genetic profiles, primary and secondary GBMs are histologically indistinguishable, and the most reliable indicator to differentiate them are mutations in the IDH1 gene [36]. A summary of the most frequent genetic alterations in primary and secondary GBMs is presented in Table 1.

<table>
<thead>
<tr>
<th>Table 1. Genetic alterations of primary and secondary Glioblastoma (GBM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary GBM</strong></td>
</tr>
<tr>
<td>[27,43–46]</td>
</tr>
</tbody>
</table>
LOH chromosome 10q (70%)
LOH chromosome 10p (50-70%)
EGFR amplification or mutation (35-45%)
TP53 mutation (27-30%)
PTEN mutation (25%)
O6-MGMT promoter methylation (42%)
TERT promoter mutation (72%)
PDGFR amplification (~7%)
MDM2 mutation (7-12%)
NF1 mutation/deletion (11%)
GLI1 mutation (5-22%)
IDH1/2 mutation (5%)
PIK3CA mutation (1%)

IDH1/2 mutation (73-85%)
TP53 mutation (65-81%)
ATRX mutation (65-71%)
LOH chromosome 10q (63%)
LOH chromosome 19q (~50%)
MGMT promoter methylation (79%)
p16INK4a deletion (~19%)
EGFR amplification (8%)
PTEN mutation (<5%)

Summary of reported genetic alterations observed in primary and secondary GBM.

In 2016, GBM classification was updated, considering the specific molecular and genetic profiles observed in the different tumors [25,47]. Based on this, GBM was classified into four subtypes: proneural, neural, classic, and mesenchymal [6,23,48–50]. The proneural group constitutes the most frequent secondary GBM and has histological features most consistent with oligodendrocytes. This subtype typically found in younger patients is associated with the best prognosis after treatment. The most frequent genetic alterations observed in this subtype of GBM are mutations in PDGFRA, IDH1, TP53, and PIK3C genes. The neural profile is characterized by TP53 mutation, EGFR amplification, and CDKN2A deletion. The histology that describes this subtype is consistent with a combination of oligodendrogial, astrocytic, and neuronal features. Also, an important composition of genes involved in nervous system development and function (NEFL, GABRA1, SYT1, and SLC12A5) and a greater degree of neuronal marker expression was observed in the neural subtype. The classic or proliferative subtype is associated with EGFR amplification (97%), LOH of chromosome 10, chromosome 7 amplification, and CDKN2A deletion (94%) and demonstrated features more consistent with astrocytes. The mesenchymal subtype of GBM is associated with a worse prognosis and evidence of a greater degree of necrosis and inflammatory components. This profile is characterized by overexpression of mesenchymal and astrocytic markers, lower expression of the tumor suppressor NF1, altered PTEN, TP53, CDKN2A, Akt genes, and the presence of mesenchymal markers (MET, CHI3L1, CD44, and MERTK). The classical and mesenchymal subtypes are associated with more aggressive high-grade gliomas, the worst prognosis compared to other profiles, and a slightly better response to aggressive therapies [23,47,48,50,51]. Table 2 summarizes the most important features of the four subtypes described above.

In addition to the molecular and genetic features, the different subtypes of GBM have also been associated with the distinct localization of the tumors in the brain. Regarding the anatomical localization of the different subtypes, it was reported that tumors belonging to proneural and neural subtypes are found in the subventricular zone (SVZ) and showed a more rapid progression and poor response to the treatment compared to those localized outside of the SVZ of the brain. On the contrary, the classical and mesenchymal GBM are localized diffusely and away from the SVZ [52]. Interestingly, a recent transcriptome analysis revealed only three subtypes of GBM, presenting strongly enriched mRNAs associated with classical, proneural, and mesenchymal subtypes. This observation suggests that the neural subtype may represent a contamination of the original samples with non-tumor cells [53].
Table 2. Features of neural, proneural, classical, and mesenchymal GBM subtypes

<table>
<thead>
<tr>
<th>GBM subtype</th>
<th>Molecular and genetic profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proneural</td>
<td>- IDH1 point mutation</td>
</tr>
<tr>
<td></td>
<td>- PDGFRA alterations</td>
</tr>
<tr>
<td></td>
<td>- TP53, DLL3, DCX, TCF4, SOX, ASCL1, OLIG2 mutations</td>
</tr>
<tr>
<td></td>
<td>- PIK3C mutation</td>
</tr>
<tr>
<td></td>
<td>- Expression of NKX2-2</td>
</tr>
<tr>
<td></td>
<td>- Associated to secondary GBM</td>
</tr>
<tr>
<td>Neural</td>
<td>- Expression of neuron markers (NEFL, GABRA, SYT1, and SLC12A5)</td>
</tr>
<tr>
<td>Classical</td>
<td>- EGFR amplification</td>
</tr>
<tr>
<td></td>
<td>- Chromosome 7 amplification</td>
</tr>
<tr>
<td></td>
<td>- LOH 10</td>
</tr>
<tr>
<td></td>
<td>- CDKN2A deletion</td>
</tr>
<tr>
<td></td>
<td>- High Notch and Sonic Hedgehog genes expression</td>
</tr>
<tr>
<td></td>
<td>- NES expression</td>
</tr>
<tr>
<td>Mesenchymal</td>
<td>- Lower expression of NF1 PTEN, TP53, CDKN2A, Akt alterations</td>
</tr>
<tr>
<td></td>
<td>- Expression of mesenchymal markers (MET, CHI3L1, CD44 and MERTK)</td>
</tr>
<tr>
<td></td>
<td>- Expression of SERPINE, TRADD, RELB, CTGF and TNFRS1A</td>
</tr>
<tr>
<td></td>
<td>- Focal deletions 17q11.2</td>
</tr>
</tbody>
</table>

Summary of features described in the literature for neural, proneural, classical, and mesenchymal GBM subtypes.

GBM treatment

In spite of the enormous efforts to develop an efficient GBM therapy, phase III studies failed to demonstrate a survival benefit in newly diagnosed and recurrent tumors. Target drugs that perform well in preclinical studies failed in the expensive phase III clinical trials in humans due to poor pharmacokinetics, the emergence of resistance pathways, GBM heterogeneity, and suboptimal clinical trial organization. Since GBM is considered an orphan disease, the enrolment in clinical trials participation is poor, and in consequence, it prevents the detection of statistically significant differences in treatment. Furthermore, the use of appropriate controls, stratification according to prognostic factors, and clinical endpoint definition are other challenges to be addressed to improve the clinical trial design. Moreover, inefficiencies in phase II to phase III transition have failed in successful drug development in GBM [54].

The heterogeneous and proliferative nature of GBM facilitates the selection of resistant subpopulations and leads to the rapid development of resistance, infiltration, and relapse [55,56]. A small population of Glioma Stem Cells (GSCs) [57–60] and the intra- and inter-tumor heterogeneity of the different subtypes of GBM [54] as well the stromal cells in the tumor microenvironment contribute to the challenges faced to treat GBM tumors successfully. The subpopulation of GSCs resides in hypoxic areas of GBM tumors due to their ability to adapt to low oxygen concentrations [61]. Consequently, hypoxic regions of the tumor contribute to tolerance to reactive oxygen species (ROS)-inducing treatments and play an essential role in therapy resistance, aggressiveness, and relapse [62,63].
On the other hand, the proper delivery, CNS permeation, and drug concentration of therapeutic drugs represent another challenge in developing an optimal GBM therapy since only selective substances such as small (less than 50 Da) and small lipophilic molecules can passively diffuse across the blood-brain-barrier (BBB) [64,65]. This is an impenetrable barrier with tight junctions and absence of fenestrations. Also, the brain uses efflux pumps at the luminal side of the BBB to recognize and remove foreign substances. Larger or hydrophilic molecules can only cross the BBB through specific transporters like the glucose transporter-1 (GLUT-1) or ATP-binding cassette (ABC) transporters [64,65].

**Current standard of care treatment**

Nowadays, there are no curative treatments for GBM, and the patients show a mean survival rate of 12-15 months, whereas the 5-years survival represents less than 5% of the GBM diagnosed patients [1,12,54,66]. The initial therapy assigned to patients with newly diagnosed GBM consists of a surgical approach in order to eliminate the primary bulk tumor. Surgical resection rarely eliminates all tumor cells since GBM is a highly diffusive, invasive, and vascularized tumor. Complete resection is currently impossible since the tumor is sometimes located in specific regions of the brain with a high level of neural compromise in function and connectivity. Consequently, the surgery could potentially affect critical areas involved in sensory processing, linguistic ability, and/or motor function. Therefore, surgical resection is not fully curative, and the infiltrating tumor cells remaining in the surrounding region can lead to recurrence, usually, months after the intervention takes place.

Since 2005, after surgery, the Stupp regimen has become the standard of care treatment, combining radiotherapy followed by the administration of 6 cycles of TMZ, a DNA-alkylating agent approved in 2005 by the Food and Drug Administration (FDA). A significant phase III clinical trial showed an improvement in the median OS compared to radiation only (14.6 months compared to 12.1 months), with a twofold increase in 2-year survival from 10.4 to 26.5% [12]. Cell death induced by TMZ treatment is promoted by increasing the cellular level of ROS [67] and controlling autophagy [68], triggering apoptosis [69], and modulating HIF-1α activity [70]. Although TMZ is part of the standard treatment for GBM, it shows unwanted toxicity, and the chemotherapeutic efficiency is significantly poor since most of the patients relapse [71]. TMZ resistance was then determined to be related to the MGMT gene that, as stated previously, encodes for a DNA enzyme that repairs the N7 and O6 positions of guanine alkylated by TMZ. Therefore, it was observed that patients with the MGMT gene silenced by promoter methylation showed a higher survival rate compared to those with hypomethylated MGMT genes exhibiting a median OS at 2 years of 46% [72–75]. Interestingly, a study shows that MGMT gene silencing by promoter methylation was evidenced in more than half of all GBM patients analyzed, highlighting the therapeutic relevance of this biomarker to decide the best treatment [76]. Also, TMZ resistance and recurrence were associated with oxidative stress [77]. As was evidenced by Zhu and colleagues (2018), TMZ-resistant glioma cells have higher levels of glutathione reductase and reduced glutathione than TMZ-sensitive cells [78]. In addition to the Stupp protocol, involving surgery followed by radio and chemotherapy, other therapeutic strategies discussed below have been developed in the past years showing promising results.

**Novel therapeutic strategies for GBM**

Since GBM is a highly vascularized tumor, anti-angiogenesis therapies have gained attention in the GBM therapeutic research field. The use of Bevacizumab, a monoclonal antibody against VEGF, showed an improvement in the progression-free survival (FPS) without amelioration in the OS after two phase III trials [79,80]. However, this therapeu-
tic approach was associated with a tumor invasiveness enhancement as a result of the induction of tumor hypoxia [81]. Bevacizumab has been considered a treatment option for patients with recurrent GBM and received full FDA approval in 2009 [54]. Notably, no other inhibitor of angiogenesis has been approved by the FDA to treat newly diagnosed or recurrent GBM apart from Bevacizumab.

The GBM microenvironment is extremely immunosuppressive, which also limits the efficacy of emerging immunotherapies. The presence of myeloid-derived suppressor cells (MDSC), regulatory T cells (Treg), cell adhesion molecules (CAM), and the recruitment of tumor-associated macrophages have been reported to contribute to an immunosuppressive microenvironment promoting immune evasion, tumor growth, invasion, angiogenesis, and resistance to chemotherapy [82–84]. Overexpression of the CD133 marker in some GBM cells has been linked to poor prognosis since it leads to immune suppression by inducing T-cell apoptosis and upregulation of Treg cells [9]. Besides, GBM is a so-called immunological cold tumor showing low immunogenicity and an immunosuppressive microenvironment with low T lymphocyte infiltration [40]. Consequently, some immunotherapeutic strategies were evaluated to assess their potential use. Immunotherapy includes cancer vaccines, modulation of specific immune checkpoint molecules using antibodies, and cellular immunotherapy with adoptive T-cell transfer (ACT) or chimeric antigen receptor (CAR) T-cell transfer.

The use of a peptide to induce an immune response against EGFRvIII was also evaluated, showing promising results in phase II clinical trials [85]. This strategy is an ideal candidate for targeted and personalized GBM therapy due to the enhanced proliferation of EGFRvIII-positive tumor cells and the lack of this variant expression in non-cancerous cells. However, the results observed in an EGFRvIII-specific peptide (CDX-110) phase III trial combined with TMZ did not show clinical benefits for patients with newly diagnosed GBM [86]. By contrast, the use of ex vivo primed dendritic cells bearing GBM-associated antigens, specific cancer stem cell markers [87] or patient-derived tumor lysates [88,89] have shown exciting results suggesting that vaccination induces a robust immune response against GBM with promising results that need to be further optimized.

T cell activation is required for specific immunological responses, and the use of antibodies that specifically abolishes the modulation of negative regulators of T cell activation is an ongoing research field in GBM treatment. This therapeutic approach includes the antibodies against PD-1 and its ligand PD-L1 and antibodies against CTLA-4. Still, the results observed in clinical trials do not show significant differences in the OS of the patients treated with these antibodies compared to that observed in the placebo group [90,91].

CAR T cells are T cells genetically modified ex vivo to express engineered chimeric antigen receptors to recognize GBM specific antigens; these cells have been investigated recently and show early promising results [92]. Interestingly, these cells are capable of recognizing GBM antigens and triggering cell lysis independently of the MHC I presentations [93]. CAR T cells genetically modified to target EGFRvIII, Her2 specifically, or IL-13Rα2 have been tested in GBM. However, due to GBM heterogeneity and the presence of an immunosuppressive microenvironment, initial clinical trial results have not shown a significant enhancement in patient survival [94]. Moreover, the use of CAR T cells can promote a cytokine-release syndrome, a systemic inflammatory response due to the activation of CAR T cells that results in the secretion of pro-inflammatory cytokines [94]. In addition to T cells, NK cells can recognize GSCs, cross the BBB [95], and be modified to express these chimeric antigen receptors [96].

Proteasome inhibitors have also been used in the treatment of GBM. A phase II study evaluating the combination of radiochemotherapy with the proteasome inhibitor Borte-
Zomib was recently reported. The results suggest that the addition of Bortezomib into the current radiochemotherapy for patients with newly diagnosed GBM was well tolerated, and the PFS and OS rates show more promising values, especially in patients with the MGMT gene promoter methylation [97]. A more recent report shows that a well-tolerated sequential treatment using Bortezomib plus TMZ promotes Th1-driven immunological responses in a group of patients showing better clinical outcomes [98].

Results from our laboratory and others showed that c-Fos, a known AP-1 transcription factor, is over-expressed in several tumors, including those from the CNS, GBM among them [99–101]. Besides its function as a transcription factor, c-Fos is able to activate the synthesis of lipids in cancer cells, and in consequence, modulates their proliferation. This activity as an activator of lipid synthesis involves the interaction of c-Fos with key enzymes of the lipid metabolism [102–107]. The interaction with these enzymes entails the N-terminal portion of the protein (NA), and its activation depends on the basic domain of the protein, known as BD. Based on these observations, and taking into consideration that c-Fos is overexpressed in CNS tumors in comparison with non-tumoral tissues in which its expression is significantly lower or at the limit of detection [101], the NA was proposed as a negative dominant of c-Fos activation of lipid synthesis. Recent results have shown that the overexpression of NA in culture and in a xenograft model of GBM impairs the proliferation of malignant cells, highlighting the capacity of c-Fos to activate lipid synthesis to be considered a new target for GBM treatment [108].

Photodynamic Therapy (PDT) was also proposed as a novel promising strategy for the treatment of different tumors [109,110], since recent evidence shows exciting results upon GBM treatment using PDT [111–113]. PDT combines light, oxygen, and a photoactivatable compound to induce a series of chemical reactions leading to cell death and tumor growth obstruction. In this field, our laboratory has observed and reported the potentiality of Zinc Phthalocyanine and one of its derivatives to impair the proliferation of GBM cells in vitro [114,115].

Tumor treating fields (TTFields) represent an innovative noninvasive antitumor strategy that involves the transcutaneous delivery to the tumor of electric fields of low intensity (1–3 V/cm) and intermediate frequency (100–300 kHz). This technique creates a significant biophysical force on dipoles and interferes with tumor cell proliferation [116]. Optune® is an example of a TTFields portable device that disrupts cell division by rapidly dividing GBM cells leading to mitotic arrest and cell death. A randomized phase III trial evidenced increased PFS and OS in the combined therapy of TTFields and standard TMZ maintenance compared to standard TMZ maintenance treatment alone in patients with newly diagnosed GBM [117]. Also, chemotherapy and TTFields treatment showed a significant increase in OS compared to chemotherapy alone [118]. Even though TTFields is a promising option approved for newly diagnosed and recurrent GBM [119], the primary obstacle is the expensive cost of the treatment, limiting its use in private clinics and institutions.

Circadian rhythms

The circadian clocks (from Latin circa: near / dies: day) temporarily regulate cell-autonomous oscillations with a 24-hour periodicity of a large array of biological processes and behaviors such as sleep/wake cycles, feeding/fasting control, metabolism, hormone secretion, and immune function [20]. The evolutionarily conserved circadian mechanism in the diverse species studied (reviewed in [120]) is made up of central and peripheral oscillators distributed in organs, tissues, and even in individual cells [20]. The suprachiasmatic nuclei (SCN) in the anterior hypothalamus harbors the master circadian clock, which is synchronized by external cues, or Zeitgebers (timer-givers) such as light or
temperature, among others to anticipate and adapt the circadian timekeeping system to the environment [121–123].

Light and the environmental illumination conditions are the strongest synchronizers of the SCN through the projections from the retina [121,124]. The master clock can coordinate circadian outputs to synchronize peripheral clocks (e.g., liver, kidney, skin, intestine, lung, pancreas, ovary, and heart) in a tissue-specific manner through the autonomic nervous and the neuroendocrine systems [122,125]. Therefore, the central clock and peripheral oscillators drive the rhythmic expression of genes to couple physiological and behavioral processes to periodic environmental changes. However, modern life characterized by increased night-time activities with prolonged artificial lighting such as rotating shift work, hypercaloric diets, shortened sleep hours, and jet lag alters endogenous homeostasis with external cues. Consequently, this misalignment characterized by loss of the correct coordination between elements of the circadian system is considered a contributing factor to the development of metabolic syndrome, inflammatory disorders, and higher cancer risk [126–129].

The molecular clock

In 2017, the Nobel Prize in Physiology and Medicine was awarded to J.C. Hall, M. Rosbash, and M.W. Young for describing the molecular clock mechanism that underlies the circadian rhythms using fruit flies as a model organism. They showed that a gene named Period encodes for a protein whose expression was regulated by a negative feedback loop [130]. Later, other proteins of the circadian machinery were identified and extended to other species to elucidate the molecular clockwork mechanism in the cell. In mammals, the molecular clock comprises the so-called transcriptional/translational feedback loops (TTFL) [120,131], including a core set of *clock genes* that encodes positive and negative regulators. The primary loop involves the positive elements *Bmal1* (aryl hydrocarbon receptor nuclear translocator-like, *Arntl*) and *Clock* (circadian locomotor output cycles kaput) and its paralogue neuronal PAS domain protein 2, *Npas2*, and the negative components *Per1/2* (Period) and *Cry1/2* (Cryptochrome) genes. During the day, the CLOCK:BMAL1 heterodimer recognizes the *E*-box sequence in *Per* and *Cry* promoters, increasing the levels of these transcripts. Then, PER and CRY proteins form a repressor complex that translocates to the nucleus and represses the CLOCK:BMAL1 activity inhibiting their expression. During the night, the repressor complex of PER and CRY is degraded, allowing CLOCK:BMAL1 to activate a new cycle of transcription and translation of approximately 24 hours [132].

Furthermore, post-translational modifications of PER and CRY proteins such as phosphorylation-dependent ubiquitination and proteasomal degradation occur in a circadian manner and regulate the subcellular localization and/or half-life of proteins contributing to the progression and beginning of a new 24-hour cycle [133–137]. In the secondary loop, the CLOCK:BMAL1 heterodimer activates the transcription of *Rev-erba/b* genes, which belong to the orphan retinoic acid receptors family. In turn, REV-ERB proteins compete with ROR receptors for binding to ROR-response elements (RORE) sequences in the *Bmal1* promoter to repress or activate its transcription, respectively [138,139]. Also, the CLOCK:BMAL1 heterodimer regulates the expression of a set of genes known as *clock-controlled genes* through *E*-box sites in their promoter regions. In this way, the circadian clock exerts its control in molecular, biochemical, and physiologic processes, including cell cycle, proliferation, metabolism, senescence, and DNA repair, among others [140–143].

Circadian disruption and its implication in cancer biology
As postulated by Hanahan and Weinberg (2011), tumor cells share common features known as hallmarks of cancer that characterize how cancerous cells disrupt cellular homeostasis promoting tumor growth. These hallmarks include sustaining signaling promoting cellular proliferation, replicative immortality, the capability of evading cell death mechanisms, the capacity to avoid growth suppressors, the ability to trigger blood vessels formation (angiogenesis), the capacity of metastasis, the deregulation of cellular energetics, and the capability to evade the anti-tumoral immunological response. The features mentioned above are associated with crucial genomic instability and inflammation, contributing to tumor development [144]. Several studies in the literature suggest tight crosstalk between the circadian clock function with tumorigenesis and cancer progression in different tumor models. It was evidenced that clock and clock-controlled genes regulate several pathways involved in cellular proliferation and growth under physiological conditions and that, when altered, may promote some of the hallmarks mentioned above of cancer, strongly suggesting that tumor cells can hijack the endogenous clock functioning to assure unrestricted proliferation, enhance the metabolism to supply their high energetics demands and adapt and modify the microenvironment to promote tumor growth [145,146].

The molecular clock can positively or negatively modulate the different cell cycle phases. Moreover, several regulators implicated in cell cycle checkpoints show daily expression patterns [147–150]. Sustaining cell signaling is considered another hallmark of cancer, and several studies suggest its connection with the circadian clock, showing that proteins related to proliferation pathways exhibited circadian patterns of expression [147,151]. In this respect, Myc oncogenic activation is also observed when deregulation of sympathetic nervous system modulation of peripheral tissues occurs [152]. Also, the circadian expression, stability, and activity of p53, one of the most studied tumor suppressors, is modulated by BMAL1 and PER2 [153–157]. Lastly, the RAS/MAPK pathway was associated with alterations in the circadian clock, in which anomalous RAS activation impairs CLOCK:BMAL1 activity and up-regulates Ink4a/Arf [158,159].

Regarding tumor metabolism, cancer cells have high energetic demands to sustain exacerbated proliferation. Circadian disruption has been associated with changes in the cellular metabolic program, modulating glucose utilization, amino acid uptake, lipogenesis, glycerophospholipid metabolism, and β-oxidation [128,160–162]. This metabolic rearrangement is known as the Warburg effect, in which metabolism mainly occurs through glycolysis, as opposed to mitochondrial oxidative phosphorylation in normal cells. Also, this phenomenon is associated with a reduction in the tricarboxylic acid (TCA) cycle activity, an increase in the synthesis of fatty acids, and an enhanced NADPH formation. Remarkably, the circadian clock regulates NADPH levels, a critical anabolic intermediate that plays a crucial role in cancer development [163]; it also regulates the expression of several genes involved in the transport and metabolism of glucose [164–166].

Since the circadian clock plays an essential role in immune system regulation, alterations in clock function have been associated with aberrant inflammation, evasion of immunological surveillance, and immune cell functionality changes leading to cancer progression [167,168]. Lastly, cell death and DNA damage response are other mechanisms involved in the recognized hallmarks of cancer associated with the circadian clock deregulation [146,169–177] (Figure 1).
Epidemiological studies

Epidemiological evidence suggests a tight correlation between circadian organization disruption and an increased incidence of specific cancer types, including prostate, breast, colon, liver, pancreas, ovary, and lung cancer [146,178–181]. In particular, epidemiological studies demonstrate that exposure to shift work for an extended period (more than 20 years) was associated with a higher risk of developing breast, prostate, and rectal cancer [182,183]. Therefore, in 2007, the International Agency for Research on Cancer (IARC) of the WHO classified the “shift work leading to a circadian disruption” as a probable human carcinogen (Group 2A) [184]. However, some studies showed that circadian disruption caused by night or shift work is not inherently carcinogenic [185,186]. Nevertheless, the aspects that link circadian disruption with an increased risk of cancer development remain unclear and need further investigation.

It should be noted that circadian disruption is influenced by the number of years of exposure, the frequency of shift work schedules, and the number of hours per week of night work in shift workers [187–190]. A pilot study postulated epigenetic modifications as a putative mechanism by which circadian rhythms are altered in shift workers based on the differences observed in methylated gene profiles in the daytime compared to night-time shift workers [188]. It was also suggested that the development of metabolic syndrome might be related to the polymorphism variations in clock genes associated with diverse chronotypes [191]. In addition to rotating work schedules, meal-timing and hypercaloric diets are other aspects of modern society that influence circadian misalignment and cancer development. Recent studies evidenced that eating dinner before 9 pm correlates with a reduced risk of prostate and breast cancer [192,193]. Similarly, mice fed with a restricted schedule showed a reduced tumor growth compared to animals fed ad libitum [194].

Although bioinformatic approaches using the TCGA database suggest a low mutation frequency of clock genes [195], it has been observed a dysregulated clock gene expression in human cancer such as epigenetic silencing by promoter methylation, dysregulation at the transcriptional or post-transcriptional level, and gene polymorphism [196–198].
and colleagues (2018) reported, after bioinformatic analysis, that 88.2% of the clock genes showed differential expression in at least one type of tumor and 94.2% of clinically actionable genes present correlation with at least one clock gene in at least five types of tumors. RNA-sequencing analysis revealed that the *Arntl2* gene was up-regulated in several tumor types and that the *Per*, *Cry*, and *ROR* genes were down-regulated in tumor tissues [195].

**Laboratory evidence**

Regarding animal models, anatomical disruption by bilateral electrolytic lesions of the master clock in the SCN showed an increase in tumor growth of implanted Glasgow osteosarcoma and pancreatic adenocarcinoma compared to sham-operated animals [199]. Besides, environmental disruption models have been implemented to investigate the impact of circadian misalignment on cancer development. In rodent models, repeated 8-hour advances in the light schedule every two days for several weeks mimic jet lag conditions similar to those experienced by humans. Jet-lagged animals showed increased growth of Glasgow osteosarcoma [200] and an enhanced incidence of lymphoma, hepatocellular carcinoma, and melanoma [152,201,202].

Moreover, *Cry* and *Per* mutant mice subjected to chronic jet lag (CJL) exhibited a higher incidence of pancreatic, kidney, and intestinal tumors [152]. Overall, these results suggest that a misalignment between the central pacemaker and peripheral oscillators, as well as with the surrounding environment, plays a crucial role in cancer development.

Although several studies suggest that clockless animals are tumor prone and clock genes have tumor-suppressive function, other findings propose more complex crosstalk between the circadian clock and cancer, including the homeostasis between stem, progenitor, and differentiated cells (reviewed in [203]). In this context, *Bmal1*+/− mice exhibited a higher incidence of lymphoma, liver, and ovarian cancer [152]. By contrast, leukemia growth *in vivo* was impaired by the lack of BMAL1, suggesting its essential role in the proliferation and stemness of acute myeloid leukemia [204].

Regarding the negative elements of the clock machinery, *Per2* genetic disruption accelerates tumor formation in different mouse models [152,153] and overexpression of *Per1* and *Per2* genes sensitizes human cancer cells to apoptosis-mediated cell death induced by DNA damage [172,205]. Similarly, irradiated mice with genetic alterations on *Per2* or *Per1/2* genes accelerate salivary gland hyperplasia, teratomas, lymphoma, liver, and ovarian cancer, suggesting that *Per* genes act as tumor suppressor genes [152,153]. However, a later study showed that *Per1* or *Per2* mutant mice do not predispose spontaneously or radiation-induced cancer [206]. Furthermore, *Cry1/2−/− p53−/−* genotype delays the onset of tumorigenesis compared to the p53 null background. The life span of these triple mutants mice was extended by ~50% after promoting apoptosis of tumor cells [207].

Since clock proteins act as transcription factors, they can directly or indirectly regulate the expression of hundreds of genes involved in pathways relevant to cancer development. Taken together, laboratory studies suggest that clock genes may have a tumor-suppressive activity or act as oncopgenes. Consequently, further investigation is needed to elucidate tissue and tumor-specific mechanisms that regulate clock function in cancer development and progression.

**Clock genes and their incidence in GBM development, progression, and prognosis**

As described above for other tumors, there is growing evidence in the literature supporting a correlation between disturbances on clock gene expression and carcinogenesis.
and progression of brain tumors. Remarkably, patient-derived GSCs and human GBM cell cultures exhibited daily rhythms on Bmal1 expression [22,208]. In addition, results from our laboratory evidenced an intrinsic cellular clock present in arrested T98G cells driving circadian rhythms in clock (Per1 and Rev-erba) and clock-controlled gene (Chok1, Pcyt-2) expression, enzyme activity, and metabolic glycerophospholipid labeling. Interestingly, T98G cells under proliferative culture conditions lost their periodicity on clock gene expression or exhibited a shortened period, whereas metabolic parameters maintained the rhythmic profile with a period close to 24 hours or longer [160]. Overall, evidence in the literature suggests that GBM cells exhibit a functional clock regulating several cellular pathways, including redox state, peroxiredoxin cycles, and other metabolic and energetic processes. Moreover, based on growing evidence, such cellular clocks seem to be involved in cancer progression and tumor cell survival. According to this, endogenous clock regulation is associated with epithelial to mesenchymal transition, angiogenesis, invasiveness, regulation of cell cycle and DNA repair system, modulation of metabolism, and apoptosis, among other well-known cancer hallmarks [209]. In this part of the review, we will focus on the evidence present in the recent literature regarding the implication of the molecular clock in the regulation of gliomagenesis and progression.

The positive arm of the molecular clock

*Bmal1* gene

BMAL1 and its binding partner CLOCK recognize the E-box motif in the promoter of clock and clock-controlled genes, activating their transcription. Since BMAL1 was shown to regulate several critical cellular processes such as cell cycle progression, lipid, and glucose metabolism, redox state, and stress response [210–212], this highlights the putative crosstalk between the molecular clock and cancer development and progression. However, controversial results about the role of BMAL1 suggest that its function is tissue and cancer-specific. In gliomas, either upregulation or downregulation of BMAL1 expression has relevant repercussions on their biology. Upregulation in the expression of BMAL1 was reported in the analysis of the TCGA database in high-grade glioma patients [208].

Additionally, BMAL1 knockdown impaired proliferation of patient-derived GSCs in culture, inducing cell cycle arrest and apoptosis as well as extending the life span and inhibiting tumor growth in a murine model [208,213]. Interestingly, targeting BMAL1 unaltered the normal neural stem cell proliferation, suggesting a critical role for this circadian regulator on GSCs growth and survival. The mechanism proposed by Dong and collaborators (2019) suggests that GSCs reprogram their metabolism through the molecular clock and epigenetic modifications since BMAL1 preferentially binds to the promoter region of genes involved in critical metabolic pathways such as those of glycolysis and TCA cycle [208].

It was also proposed that BMAL1 may act as a tumor suppressor in GBM cell growth. For instance, Jung and colleagues (2013) reported that BMAL1 overexpression impairs glioma invasiveness by blocking the PI3K/AKT/matrix metalloproteinase-2 signaling pathway [214]. In concordance with this, BMAL1 overexpression significantly decreases U-87MG cell viability (a cellular model of GBM) and Cyclin B1 levels, which play a critical role in the G2/M transition cell cycle. Also, the expression of pro-apoptotic proteins was increased while the anti-apoptotic protein BCL-2 level decreased, suggesting that BMAL1 may operate as a tumor-suppressor in U-87MG cell cultures. Glioma migration and invasion were also reduced after ectopic expression of BMAL1, leading to down-regulation of p-AKT and MMP-9 signaling pathways [215]. Similar to the observations described above, results obtained recently by Wagner and colleagues (2021) show that
the downregulation of Bmal1 expression is associated with a more aggressive form of the tumor. In this study, a cell line isolated from a malignant peripheral nerve sheath tumor generated in NPcis mice (an animal model for the human neurofibromatosis type I) was used as a glioma model after being injected into C57BL/6 animals and tumor growth evaluated. The results showed that, after the knockdown of Bmal1 using CRISPR/Cas9, tumors grew faster than those from control cells [21]. Suliman Khan and colleagues (2019) identified oncogenes and tumor suppressor genes that show significant variations in their expression in brain tissues from animals exposed to a CJL protocol. Interestingly, this study uses Bmal1−/− animals and suggests that expression of some of these genes is associated with the clock, highlighting the link between circadian disruption by CJL and the risk of glioma development [216]. More studies are needed to fully comprehend the biological importance of the circadian transcriptional regulator BMAL1 in the genesis and progression of brain tumors.

Clock gene

As it was reported for its binding partner BMAL1, TCGA database analysis revealed that the Clock gene, located at 4q12 chromosomal region, is amplified in ~5 % of GBM patients [213,217], and high-grade gliomas exhibited an increased expression of CLOCK compared to low-grade glioma or non-tumor cells [218–220]. An exploratory study carried out by Madden and collaborators found that CLOCK was overexpressed in tumors and that a single nucleotide polymorphism (rs7698022) present in the Clock gene was correlated with mortality in high-grade glioma patients [220]. A report in the literature indicates that CLOCK explicitly modulates the proliferation and cell death after irradiation in U-87MG cells. After Clock silencing, a reduction in proliferation and induction of apoptosis was observed in glioma cells. This phenomenon was associated with a downregulation of c-Myc and Cyclin B1 and upregulation of p53 related genes. These results highlight the anti-apoptotic modulation of CLOCK in glioma cells [221]. In human GSCs, CLOCK was proposed as a critical regulator of metabolism required for optimal GSC growth and survival since CLOCK depletion impaired GSCs self-renewal, reduced enzyme expression involved in glycolysis and TCA, and triggered cell cycle arrest and apoptosis [208,213]. These results agree with the findings described above by Dong and collaborators (2019) that evidence the crucial role of BMAL1 and CLOCK in tumor metabolism and stemness maintenance [208].

Additionally, Chen and colleagues (2020) suggested that CLOCK is implicated in the modulation of immune-suppressive microglia infiltration into the tumor microenvironment, seemingly by regulating the expression of the chemokine OLFML3. Interestingly, in the results obtained using an in vivo model of GBM, it was observed that downregulating the expression of CLOCK or OLFML3 shows an extension in the lifespan of mice compared to the control group [213]. More evidence suggests that CLOCK has a tumor-promoting function in gliomas. Li and collaborators (2013) showed in a fascinating study that high expression of CLOCK observed in high-grade gliomas tissues and GBM cell lines is associated with an attenuated miR-124 expression. This miRNA specifically targets the 3’UTR of Clock mRNA, and it was previously reported to impair cell proliferation and migration of tumor cells. Remarkably, CLOCK might promote the proliferation and migration of glioma cells through the NF-kB signaling pathway [219]. By contrast, the report from Wang and collaborators (2021) shows that CLOCK is downregulated in GBM samples [222].

Overall, the results discussed above suggest that CLOCK may promote tumor proliferation in different glioma models and play a critical role as a regulator of tumor metabolism. Considering this evidence, targeting the circadian clock by “adjusting the CLOCK” could be a promising strategy for GBM treatment, especially to impair GSC growth.
The negative arm of the molecular clock

**Period 1 gene**

*Per1* encodes for PER1 protein, a negative element of the circadian transcriptional machinery. Early studies from Wang’s laboratory showed that PER1 expression is lower in high-grade gliomas than in the surrounding non-tumor tissues. This study suggested that the deregulation in PER1 expression allows glioma cells to proliferate and survive, as this was related to a disruption of the clock function [223]. Similar results recently showed a reduction in PER1 expression in high-grade gliomas [222]. In agreement with these observations, tumors generated by injection of cells isolated from a malignant peripheral nerve sheath tumor exhibited lower *Per1* mRNA levels than normal tissue [21]. One possible explanation linking the low levels of *Per1* mRNA and protein with a higher tumor malignancy may be likely associated with the opposite relationship found between its expression and the phospholipid biosynthesis required for the genesis of the new membranes and other essential processes during cell growth and proliferation, as was observed in a non-malignant fibroblast cell line [161].

On the other hand, the analysis performed by Madden and collaborators (2014) found overexpression of *PER1* and identified a *PER1* variant (rs2289591) associated with glioma risk and, similar to the CLOCK variant described above, it was associated with mortality in high-grade glioma patients [220]. Interestingly, *Per1* expression was related to the radiosensitivity of gliomas in culture. In this sense, *Per1* downregulation attenuated U343 glioma cell radiosensitivity, decreasing the apoptosis of irradiated tumor cells. Since *PER1* knockdown decreased the levels of CHK2 and p53 proteins, critical checkpoints in DNA damage, the authors suggest that *PER1*, as a tumor suppressor gene, modulate the p53 pathway and, in consequence, influences p53 levels with a direct effect on apoptosis promotion and proliferation inhibition [224]. Similarly, high expression of *Per1* correlated with increased radiosensitivity in glioma cells of a rat model, while this phenomenon was not evidenced in non-tumor tissues. This study observed that *Per1* levels show a circadian expression pattern in both normal and tumor tissues. However, glioma tissues evidenced a 12-hours periodicity on *Per1* expression while normal tissues displayed oscillations with a period close to 24 hours. Like the previously described report, the author highlights the tumor suppressor role of *PER1* in gliomas, showing that its expression is related to cell cycle arrest and enhanced x-ray sensitivity [225]. Also, findings from our laboratory demonstrated a 28 and 16-hours rhythmicity on *Per1* mRNA levels in arrested and proliferative T98G cultured cells, respectively [160]. A recent report by Gao and collaborators (2021) shows that the IDH1 mutation (R132H) is associated with a reduction in GBM cell proliferation as well as with the modification in clock gene levels, including a decrease in the expression of *PER1* [226]. Besides the downregulation of *Per1* levels observed in gliomas, these results suggest that tumor cells may display aberrant oscillations on *Per1* expression, influencing cell proliferation and tumor survival.

**Period 2 gene**

*PER2* protein expression in gliomas has also been reported to be disturbed in comparison with normal brain tissues. Early results showed that *PER2* expression was significantly lower compared to non-glioma cells, bringing out differences in the expression of clock genes between normal and malignant brain tissues [223]. Later and in concordance with the previous report, Wang and collaborators (2014) analyzed the expression of *PER2* in glioma samples by immunohistochemistry and found a significant reduction in *PER2* expression associated with high-grade gliomas and higher expression of EGFR and PCNA. Additionally, the authors proposed that promoter methylation or cell signaling
pathways disruption may influence PER2 expression in tumor tissues [227]. In the same line, PER2 was found to be downregulated in samples from the TCGA database [222], and deregulation in PER2 tumor expression was associated with higher mortality in the cohort of glioma patients [228]. Similar to that observed for PER1, PER2 expression was associated with effectiveness in radiotherapy, again supporting the hypothesis that both genes are tumor suppressors [225].

A crucial role of PER2 in gliomagenesis was recently informed. Per2 mRNA and protein levels were reported to be downregulated in GSCs, and its overexpression impairs its proliferation through the cell cycle, arresting them in G0/G1 phase. The authors suggest that since PER2 targets the Wnt/β-catenin signaling pathway in GSCs, the downregulation of critical proteins involved in invasiveness and stemness of GSCs, such as Wnt7b, β-catenin, MMP2, MMP9, and c-Myc, may explain the tumor suppressor role of PER2 in gliomas [229]. The IDH1 R132H mutation was also associated with a decrease in protein levels for PER2 [226].

**Period 3 gene**

PER3 expression decrease in gliomas has been observed and related to higher mortality [220,228]. Wang and collaborators (2021) showed that the analysis of TCGA samples indicates a reduced PER3 expression in GBM samples [222]. Moreover, IDH1 R132H mutation was associated with a reduction in PER3 expression level [226]. The above observations suggest that similar to PER1 and PER2, PER3 could be crucial to gliomagenesis, acting as a tumor suppressor gene. Nevertheless, the role of PER3 in gliomagenesis and progression needs to be further investigated.

**Cryptochrome 1 gene**

The circadian proteins CRY and PER form a repressor complex that inhibits their transcription, and that of other clock-controlled genes once translocated to the nucleus and represses the CLOCK:BMAL1 heterodimer transcriptional activity. Therefore, CRY as well as PER proteins are critical factors in the maintenance of cellular circadian homeostasis. A study of 69 sample patients evidenced a downregulated expression of Cry1 in glioma tissues compared with non-tumor cells [230]. Conversely, TCGA database analysis reported higher levels of Cry1 in GBM patients than in normal brains [220,222]. In U-87MG cell cultures, mutations in the IDH1 gene significantly correlated with a downregulated Cry1 expression compared to control cells. This study model proposed that IDH1 mutation affects glioma proliferation by altering clock gene expression through the TGF-β/Smad signaling pathway [226]. Also, the role of Cry1 on glioma biology has been evidenced in experimental models of Cry1/2 double knockout mice subjected to CJL conditions. These results suggested a link between clock genes and glioma-related genes as well as the implication of lighting conditions in carcinogenesis [216]. Lastly, recent research proposed the pharmacological modulation of the circadian clock as a novel strategy for GBM treatment. KL001 is a synthetic agonist that stabilizes CRY protein levels, preventing their degradation. Dong’s laboratory showed that KL001 treatment impaired GSC proliferation and decreased stem cell markers expression [208].

**Cryptochrome 2 gene**

In glioma tissues, expression of Cry2 was attenuated compared to healthy samples [230,231] and correlated with higher mortality [228]. However, findings of irradiated
glioma cells on a rat model showed a correlation between the increased expression of Cry2 and increased cell proliferation and decreased apoptosis. As mentioned above, for Per1 expression, disturbances on rhythmic expression of Cry2 were observed in glioma tissues with a period of 8 hours compared to 24-hours periodicity displayed by normal brain samples suggesting that an altered rhythmic expression of Cry2 influences sensitivity to irradiation on gliomas cells [232]. Similar to results observed on Cry1 expression, IDH1 mutated U-87MG cells showed lower levels of Cry2 than wild-type cells with implications in tumor proliferation [226]. In view of the findings described, it can be inferred that the expression of the negative circadian regulator Cry2 is altered in gliomas tissues. Nevertheless, further investigation is needed to elucidate the key role of Cry2 in GBM development.

Rev-erb genes

NR1D1 and NR1D2 genes encode for the nuclear receptors known as REV-ERBα and REV-ERBβ, respectively. These nuclear receptors play critical functions in circadian rhythms, lipid and glucose metabolism, tumorigenesis, and inflammation and have been proposed to act as the molecular clock components linking the circadian clock with the cellular metabolism [233–237]. In glioma tissues, REVERBβ levels are lower compared to non-glioma tissues [228]. Conversely, Yu and colleagues (2018) reported a high expression of REVERBβ in GBM tissues and cell lines which was not observed in primary human astrocytes. Results from this laboratory suggested that NR1D2 is involved in the migration and invasion of glioma cells through the receptor tyrosine kinase AXL [238]. Synthetic agonists of the nuclear receptors REV-ERBs (SR9009 and SR9011) have been selectively lethal in different cancer cell lines, including GBM. For instance, T98G cells showed the highest response to SR9009 treatment in a time window from 18 to 30 hours post synchronization with dexamethasone [239]. Since SR9009 can cross the BBB [234], REV-ERBs agonists have emerged as an interesting approach to GBM treatment. In vivo experiments showed that SR9009 treatment impaired glioma growth and improved mice survival [213,240]. Remarkably, SR9009 efficacy to reduce tumor growth was similar to that observed with the current standard of care treatment, TMZ. Sulli and colleagues (2018) proposed that the pharmacological modulation of the circadian clock by REV-ERBs agonists impair tumor proliferation, inhibiting de novo lipogenesis and autophagy, which are well-known hallmarks of tumor cells [240]. Further evidence show alterations in tumor metabolism after SR9009 treatment, as is the case of T98G cells that increase the average size of lipid droplets [239] and GSCs, which reduce the expression of genes involved in glycolysis, TCA cycle, and lipid metabolism [208]. Considering that REV-ERBs inhibit Bmal1 expression, agonists of these nuclear receptors could be regarded as an exciting novel approach to target Bmal1, which has been shown to have tumor-promoting features in GBM models as discussed above.

Other clock pathways related genes

Other genes associated with the molecular clock were found to correlate with gliomagenesis and progression. NPAS2 is a protein coded by the npas2 gene that heterodimerizes with BMAL1, and its expression in gliomas was associated with patients having poor outcomes and high mortality [220]. RORα and RORβ, which modulate the transcription of Bmal1, were observed to be downregulated in gliomas, and this expression profile was prognostic in a cohort of glioma-diagnosed patients suggesting that its function is associated to the tumor genesis and progression [228]. TIMELESS is a protein belonging to the clock machinery, its activity regulates Clock, Per, and Cry gene expression and interacts with S-phase checkpoint proteins, having a crucial role in modulating the cell cycle. Recent work from Wang and Chen (2018) evidenced that TIMELESS is overexpressed in high-grade gliomas compared to low-grade glioma and non-pathological tissues. The authors suggested that this imbalance in the expression of the timeless gene
results in the abnormal progression of circadian rhythms and gliomagenesis promotion [241]. Similar to the former study, higher TIMELESS expression was observed in GBM compared to low-grade gliomas. Moreover, silencing of TIMELESS by siRNA leads to cell cycle arrest in G0 phase and cell proliferation impairment, again showing the importance of TIMELESS on GBM promotion by modulation of cell cycle and proliferation [231].

**Chronotherapy as a promising strategy for GBM treatment**

Based on recent findings discussed in this review, the evidence accumulated to date clearly shows that the biological timekeeping system is intricately connected with cancer development and progression. The above-discussed literature highlights that more profound knowledge regarding the circadian modulation on cancer biology could either improve tumor treatment or develop new therapeutic strategies. Besides the promising agonists of the clock proteins that show antitumor activity on brain tumors, chronotherapy is a growing field of research that aims to improve the efficacy of current GBM treatment. Chronotherapy is defined as the drug delivery schedule based on patients’ circadian rhythms, giving the drug administration timing an important role in therapy. This approach aims to determine the optimal time of the day to perform the treatment and improve outcomes with the most effective drug concentrations (not necessarily the highest dose used), and reduce drug toxicity and side effects.

Early evidence in this area showed that the highest response to TMZ treatment occurs near the peak of Bmal1-luc expression in a murine cellular model of GBM in culture. Moreover, phosphorylation of the histone H2AX and activation of apoptosis after TMZ treatment displayed a circadian pattern that correlates with that observed for Bmal1 expression. Remarkably, the caspase activity oscillation induced by the DNA alkylator is abolished in Bmal1 knockout cells by CRISPR/Cas9 technology compared to control cells, suggesting a mechanism dependent on the Bmal1 clock gene expression [22]. In the same way, GBM T98G cells exhibited a significant temporal susceptibility response to the proteasome inhibitor Bortezomib which is used in advanced stages of GBM treatment. Bortezomib-treated cells display the highest susceptibility in a time window ranging from 12 to 24 hours post synchronization, times in which the cellular redox state is increased. Interestingly, the circadian clock disruption through Bmal1 knockdown on T98G cells exhibited a marked 6 hours-phase advance in the temporal response to Bortezomib compared to control cells [160]. Also, T98G cells treated with the synthetic REV-ERB agonist SR9009 showed significant differences in cell viability across time, exhibiting the lowest response to the treatment at 6 h post synchronization. Moreover, the combined treatment of SR9009 with Bortezomib further potentiates their cytotoxic effects, clearly demonstrating a significant synergic impact of the drug combination. Since both chemotherapeutics act on different cellular targets, Bortezomib inhibits the proteasome activity while SR9009 acts on the clock-related cellular metabolism, the combined treatment should be considered as a chemotherapeutic approach for GBM cells [239].

Another *in vitro* study reported a rhythmic pattern of p38 MAPK activity in glial cells while its levels were arrhythmic and high in IM3 glioma cells. VX-745, an inhibitor of p38 MAPK, shows an improvement to reduce glioma invasion at a specific time of day after serum shock on IM3 glioma cells [242].

Recent results from our laboratory showed significant differences in Bortezomib efficacy on tumor-bearing mice when the drug was administered at the beginning of the day in the light phase or at night in the dark. We showed that a chemotherapeutic scheme in which a high dose (1.5 mg/kg) of Bortezomib was administered wholly inhibited tumor growth at both times; whereas a low dose of Bortezomib (0.5 mg/kg) displayed higher efficacy to impair tumor growth when delivered at night compared to diurnal treatment [21] (Figure 2).
Figure 2. Tumor growth inhibition (TGI) of glioma xenografts by chrono-chemotherapy using Bortezomib. Schematic representation of the results recently reported by Wagner and collaborators (2021) [21].

A recent retrospective study on GBM patients reported that the administration of TMZ in the morning was correlated with an increased OS in MGMT-methylated patients compared with those subjected to evening treatment. The median OS was improved by 6 months in MGMT-methylated patients who received the alkylating chemotherapeutic in the morning. Remarkably, these differences were not observed in the MGMT-unmethylated GBM cohort, a result that was expected since the unmethylated MGMT gene is usually a sign of resistance to TMZ treatment [243]. Interestingly, the authors emphasize that therapy in the morning may improve survival in older patients (over 60 years old) who tend to show earlier chronotypes [244]. Similarly, our results showed a higher tumor growth inhibition when Bortezomib was applied at night, a time at which mice exhibit the active phase of locomotor activity and feeding habits. These results strongly suggest that the chronotype will be a critical factor to be considered in chronotherapy research.

Since DNA damage repair oscillates, TMZ could be an ideal candidate to be administered at specific times of the day. Consequently, a phase II clinical trial (NCT02781792) is going on with 40 patients diagnosed with high-grade gliomas. Randomized patients receive the TMZ in the morning (before 10:00 am) or in the evening (after 08:00 pm). The results, including adverse effects and patient survival, are expected to be available in November 2022.

Table 3. Evidence of dysregulated clock gene expression in GBM

<table>
<thead>
<tr>
<th>Gene</th>
<th>Evidence of gene deregulation associated with gliomagenesis - Evidence of potentiality for therapeutic targeting</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLOCK</td>
<td>[208,213,217–222,228]</td>
</tr>
<tr>
<td>BMAL1</td>
<td>[21,22,160,208,213,214,216,245]</td>
</tr>
<tr>
<td>PER1</td>
<td>[21,160,220,223–226,228,246]</td>
</tr>
<tr>
<td>PER2</td>
<td>[223,225–229,246]</td>
</tr>
<tr>
<td>PER3</td>
<td>[220,222,226,228]</td>
</tr>
<tr>
<td>CRY1</td>
<td>[208,216,220,226,230,246]</td>
</tr>
<tr>
<td>CRY2</td>
<td>[216,220,226,228,230,232]</td>
</tr>
<tr>
<td>NPAS2</td>
<td>[220,246]</td>
</tr>
<tr>
<td>REV-ERB</td>
<td>[208,213,228,238–240]</td>
</tr>
<tr>
<td>ROR α</td>
<td>[228]</td>
</tr>
<tr>
<td>ROR β</td>
<td>[228]</td>
</tr>
</tbody>
</table>
Summary of references available in the literature regarding the implication of circadian clock genes in the biology of GBM.

Concluding remarks and future directions

GBM is considered one of the most aggressive tumors showing a bad prognosis after diagnosis. Even after great efforts made by several research groups worldwide, non-significant changes were obtained in the last years that improve the OS of patients diagnosed with GBM. Recent results obtained in the chronobiology field highlight the crucial importance of circadian modulation on cancer biology and how tumor cells can reprogram biological clocks to ensure their survival. Notably, recent advances in the field of gliomas and particularly in GBM unmask that the circadian clockwork of hosts in animal models and then their translational use in patients under clinical trials should be considered as new targets for the rational design of novel therapeutic strategies or to improve the current therapies that aim to abolish/impair tumor growth. Chronotherapy considers the biological rhythms present in the cells to determine the best time of drug administration to improve the therapeutic outcome and diminish the undesirable side effects. In addition, it must be considered that the biological clock of the whole organism drives the circadian rhythms in the immune system required to attack the tumor, in the cellular metabolism and bioenergetics, in the drug pharmacokinetics and pharmacodynamics, and detoxification mechanisms after chemotherapy among many other time-controlled aspects, that somehow temporally regulate the pathophysiological state and susceptibility of individuals once they are confronted to a disease such as cancer.

The recent reports discussed in this review show that despite promising results with chronotherapy schedules on different types of cancer, further research is needed in the context of GBM to implement different delivery schemes based on circadian rhythms for new antitumor drugs such as REV-ERB and CRY agonists, together with or as an alternative to already approved TMZ, in order to improve the survival of patients diagnosed with this devastating disease.

Author Contributions: P.M.W. Writing – Original Draft Preparation, Writing – Review & Editing; C.G.P. Writing – Original Draft Preparation, Writing – Review & Editing, Funding acquisition B.L.C. Review & Editing, Funding acquisition; M.E.G. Review & Editing, Funding acquisition. All authors approved the final version.

Funding: The work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica, Secretaría de Ciencia, Tecnología e Innovación Productiva de Argentina (PICT 2015-3702 and PICT 2017-2761 to B.L.C., PICT 2015-2986 to C.G.P, PICT 2016-0187 and PICT 2017-0631 to M.E.G.), Fundación Florencio Fiorini to C.G.P., Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Secretaría de Ciencia y Tecnología, Universidad Nacional de Córdoba (SECyT).

Acknowledgments: All authors would like to thank Dra. Ana C. Racca for critical reading of the manuscript. P.M.W. thanks CONICET for her fellowship. C.G.P and M.E.G. are career members and B.L.C contracted career member of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET- Argentina) and professors at the Universidad Nacional de Córdoba.
Conflicts of Interest: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

References


103. Cardozo Gizzi, A.M.; Caputto, B.L. Mechanistic insights into the nongenomic regulation of phospholipid synthesizing enzymes. IIIBM Life 2013, 65, 584–592.


