HHS Public Access

Author manuscript

J Biol Rhythms. Author manuscript; available in PMC 2019 March 11.

Published in final edited form as:

J Biol Rhythms. 2017 April ; 32(2): 121–129. doi:10.1177/0748730417696788.

Cell-intrinsic, Bmal1-dependent circadian regulation of temozolomide sensitivity in glioblastoma

Emily A. Slat[1,2,*], **Jasmin Sponagel**[2,*], **Luciano Marpegan**[2,3,*], **Tatiana Simon**[2], **Najla Kfoury**[1], **Albert Kim**[4], **Andrea Binz**[2], **Erik D. Herzog**[2,5], and **Joshua B. Rubin**[1,5]

[1]Department of Pediatrics, Washington University School of Medicine, St Louis, MO, 63110.

[2]Department of Biology, Washington University in St Louis, St Louis, MO 63130-4899.

[3]National University of Quilmes/CONICET, Buenos Aires, Argentina,

[4]Department of Neurosurgery, Washington University School of Medicine, St Louis, MO, 63110,

[5]Department of Neuroscience, Washington University School of Medicine, St Louis, MO, 63110

Abstract

The safety and efficacy of chemotherapeutics can vary as a function of the time of their delivery during the day. This study aimed to improve the treatment of glioblastoma (GBM), the most common brain cancer, by testing whether the efficacy of the DNA alkylator temozolomide (TMZ) varies with the time of its administration. We found cell-intrinsic, daily rhythms in both human and mouse GBM cells. Circadian time of treatment impacted TMZ sensitivity of murine GBM tumor cells *in vitro.* The maximum TMZ-induced DNA damage response, activation of apoptosis and growth inhibition occurred near the daily peak in expression of the core clock gene *Bmal1.* Deletion of *Bmal1* (*Arntl*) abolished circadian rhythms in gene expression and TMZ-induced activation of apoptosis and growth inhibition. These data indicate that tumor cell-intrinsic circadian rhythms are common to GBM tumors and can regulate TMZ cytotoxicity. Optimization of GBM treatment by timing TMZ administration to daily rhythms should be evaluated in prospective clinical trials.

Keywords

cancer; Period2 gene; Bmal1 gene; GBM; H2AX; DNA repair; astrocytoma

Introduction

Glioblastoma (GBM) is the most common and aggressive malignant primary brain tumor in adults. Despite extensive research and clinical trials, median survival remains about 15 months (Stupp et al., 2009). Therefore, all opportunities to improve outcomes should be pursued. In 2005, a landmark paper demonstrated a 2.5-month increase in median survival

Corresponding Authors: Joshua B. Rubin, MD, PhD, Campus Box 8208, 660 South Euclid Ave, St. Louis, MO 63110, (Tel) 314-286-2790, (Fax) 314-286-2892, rubin_j@kids.wustl.edu, Erik D. Herzog, PhD, Campus Box 1137, Department of Biology, Washington University, St. Louis, MO 63130-4899, (Tel) 314-935-8635, (Fax) 314-935-4432, herzog@wustl.edu.

*These authors contributed equally.

and an increase in the 2-year survival rate (10% to 27%) by treating GBM with TMZ chemotherapy during and after radiotherapy (Stupp et al., 2005). Since then, TMZ has been a standard component of GBM treatment. Investigating opportunities to enhance the anti-tumor efficacy of TMZ has significant potential for expedited translation to patients.

The efficacy and tolerability of cancer chemotherapeutics at different times of day has been investigated. Drug administration based on daily biological rhythms is known as chronotherapy. Chrono-chemotherapy has increased 5-year survival rates in acute lymphoblastic leukemia and increased the objective response rate in colorectal cancer while reducing toxicities (Levi et al., 1995; Schmiegelow et al., 1997). Chronotherapy has never been applied to the treatment of brain tumors. Standard administration of TMZ is a single, daily dose for 5 consecutive days at the start of every 28-day treatment cycle (Newlands et al., 1992; Stevens et al., 1987). The time of day of TMZ administration has not been tested as an independent variable in analyses of outcome or toxicity. Its oral administration and 1.8 h half-life in plasma (Beale et al., 1999) make TMZ an ideal candidate for chronotherapy.

Daily rhythms in sleep, hormone release and other processes can affect drug efficacy and toxicity (Vitaterna et al., 2001). The foundation of daily rhythms is a molecular clock that generates near 24 h oscillations in gene expression through BMAL1- and CLOCK-mediated transcription of circadian genes including the *Period* (*Per1, Per2* and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) loci. With a delay of about 12 h, the PER and CRY proteins accumulate and repress BMAL1/CLOCK-dependent transcription of genes including of *Per* and *Cry* (Reppert and Weaver, 2002). This feedback loop creates daily oscillations in approximately 50% of genes (Zhang et al., 2014). Transcription of *Bmal1* (*Arntl*) peaks in anti-phase to the *Period* genes (Nakajima et al., 2004; Preitner et al., 2002; Sato et al., 2004) and loss of *Bmal1* abolishes molecular and cellular circadian rhythms (Bunger et al., 2000).

We aimed to test whether GBM cells exhibit intrinsic circadian rhythms in gene expression and their response to chemotherapy. Using an *in vitro* mouse model of GBM, we examined the role of *Bmal1* in daily rhythms in Per2 expression and TMZ-induced DNA damage.

Materials and Methods

Human GBM cell culture:

Low passage primary human GBM cells were obtained and utilized in accordance with a Washington University Institutional Review Board (IRB) approved Human Studies Protocol (#201102299). They were maintained as adherent cultures on laminin (Sigma L2020) coated tissue culture plates in RHBA media (Clonetech), supplemented with 20 ng/ml epidermal growth factor (EGF, Sigma) and 20 ng/ml basic fibroblast growth factor (bFGF, Chemicon) as described (Barone et al., 2014).

The Cancer Genome Atlas (TCGA) Queries:

Details of core clock gene mutations in GBM were obtained by querying TCGA through cbioportal.org. Three datasets were queried: TCGA provisional with 604 samples, TCGA 2013 with 206 samples and TCGA 2008 with 580 samples. We searched for mutations-only

in the following genes: (*CLOCK, ARNTL, ARNTL2, NPAS2, CRY1, CRY2, PER1, PER2, PER3, CSNK1D, CSNK1E, RORA, RORB, RORC, NR1D1 AND NR1D2).*

Clock gene sequencing:

Total RNA was isolated using the RNeasy Mini system and then treated with DNase I according to the manufacturer's instructions (Qiagen). 1.5 μg of total RNA from cultured human GBM cells was reverse transcribed with the SuperScript III using random hexamers and Oligo(dT) (Invitrogen). 50 ng of this reaction served as template for quantitative real-time RT-PCR analysis using iTaq Universal SYBR Green Supermix PCR reagents (Biorad). Primers (Table S1) were designed by DS Gene software (Accelyrs, Inc. San Diego, CA. USA) to include the specific mutation of interest identified from the analysis of clock gene mutations in the TCGA database and had the following parameters: 18–27 bases, product 100–200 bp and product melting temperatures within 66°C–78°C. PCR reaction products were cleaned using the QIAquick PCR Purification Kit according to the manufacturer's instruction. Gene amplicons were verified by size on a DNA agarose gel. Samples were then sequenced for mutation(s) using Genewiz services (South Plainfield, NJ).

Animals:

Animals were used in accordance with National Institutes of Health guidelines following protocols approved by the Washington University Animal Studies Committee. NCR nude mice (Taconic Farms, Inc., NY) and *Nf1*$^{flox/flox}$*;GFAP-Cre* were housed under a 7am lights-on, 7pm lights-off schedule.

Male Astrocyte Cultures:

As a cellular model of mesenchymal GBM (mes-GBM), primary cultures of astrocytes were prepared from male, postnatal day 1 *Nf1flox/flox;GFAP-Cre* mice and rendered null for p53 function as previously described (Sun et al., 2014; Warrington et al., 2007). Due to increased risk for mesenchymal GBM in male mice and humans (Sun et al., 2014), we limited our studies to male mes-GBM astrocytes.

Expression of CRISPR-Cas9 expression vectors:

The Genome Engineering Center at Washington University in St. Louis designed and cloned six guide RNAs targeting and disrupting the *Bmal1* locus (*Bmal1 KO*). *Bmal1* WT and KO cultures were used at the same passage number in parallel experiments.

Expression of Per2-luc and Bmal1-luc reporters:

We infected astrocyte cultures with lentiviral reporter constructs expressing firefly luciferase driven by the mouse *Bmal1* (*Bmal1-luc)* (Liu et al., 2008; Zhang et al., 2009) or *Period2* (*Per2-luc*) (Ramanathan et al., 2012) promoters (generous gifts of Dr. Andrew Liu (University of Memphis)).

Expression of Casp-luc reporter:

Mes-GBM astrocyte cultures were transfected with the Caspase-3/7-luciferase plasmid (*Casp-luc*; generous gift of Dr. Alnawaz Rehemtulla (University of Michigan)) (Galban et

al., 2013) using Fugene 6 (Promega, Madison, WI). Stable lines were selected with 400 μg/ml G418 (Santa Cruz Biotechnology, Dallas, TX) and maintained in CO_2- buffered *DMEM* supplemented with 10% FBS, 1% penicillin/streptomycin. We performed 4 independently plated experiments with *Bmal1* WT and *Bmal1* KO cultures from 2 lots of mes-GBM cells. Of the 8 independent cultures recorded, 1 *Bmal1* WT and 1 *Bmal1* KO cultures were excluded because they did not respond to TMZ.

γH2AX immunofluorescence staining and quantification:

γH2AX staining measured DNA repair response in mes-GBM astrocytes. Cells plated on poly-D-lysine-coated glass coverslips were fixed with 4% paraformaldehyde, permeabilized with Triton-X-100, incubated with mouse anti-phospho-S139 H2AX primary antibody (1:800; Molecular Probes) for 3 h at 37°C, and incubated with Alexa Fluor 568 Donkey anti-mouse IgG (1:10,000; Life Technologies, Carlsbad, CA) for 1 h at room temperature (RT). Astrocyte nuclei were stained with DAPI (Life Technologies, Carlsbad, CA). Nuclear γH2AX staining intensity was quantified by two individuals blinded to treatment conditions by ImageJ analysis of integrated density of fluorescent images. Staining across - high-powered fields of view per coverslip was averaged across two coverslips per treatment condition. Thresholds for positive staining were defined by the intensity distributions of TMZ-versus DMSO-treated cells.

Bioluminescence recordings in vitro:

We detected light from clock gene reporters (*Bmal1-luc* or *Per2-luc*) with photomultiplier tubes (HC135–11; Hamamatsu Corp.) in light-tight incubators (Beaule et al., 2011; Marpegan et al., 2009; Prolo et al., 2005). We integrated bioluminescence every 6 min over a 4–5 day experimental period. During recordings, we sealed lids with vacuum grease and maintained cultures at 34°C in bioluminescence recording medium (HEPES-buffered DMEM supplemented with 10% FBS, B27 (1X; Gibco/Life Technologies, Carlsbad, CA) and 0.1 mM D-luciferin (Xenogen, Alameda, CA)), as previously reported (Marpegan et al., 2009). Bioluminescence from GBM cultures was recorded with a low light imaging system (Stanford Photonics) consisting of a light-tight incubator coupled to ICCD camera (XR/Mega10-Z, Stanford Photonics) controlled with Micro-Manager software (Edelstein et al., 2014). Cells were plated in laminin coated 96-well plates (2.5×10^4cells/well) and maintained in a mixture of 50% bioluminescence recording medium and 50% RHBA supplemented with 20 ng/ml EGF and 20 ng/ml bFGF. Images were obtained by integrating light every three minutes and then processed using ImageJ software to obtain the average signal intensity for each well every half hour. In *Casp-luc* experiments, we entrained cultures by shifting the temperature between 30°C and 34°C every 12 hours for 48 hours (Buhr et al., 2010).

Statistical Analysis:

Comparisons between treatments and genotypes (GraphPad Prism version 6.0, GraphPad, San Diego, CA) considered astrocytes derived from a single litter of mice as a single biological replicate. We performed statistical analyses on at least 3 biological replicates per experiment, with each lot derived from an independent litter of pups. Circadian period of

bioluminescence recordings was analyzed with Chronostar V2.0 software (gift of A. Kramer and B. Maier, Charite).

Results

Human GBM cells are circadian

To determine whether GBM cells have intrinsic daily rhythms, we characterized expression of the core circadian gene, *BMAL1* (also called *ARNTL*), in human primary low-passage GBM cell lines. Using a transgenic bioluminescent reporter, we recorded *BMAL1* promoter activity continuously for 5 days from GBM cells cultured from 5 patients (Figure 1). We found daily rhythms in BMAL1 expression from all 5 lines with circadian periods ranging from 22.5–27.8 h. Because altered clock gene expression in human cancers has implicated circadian rhythms in tumor progression (Stevens, 2005; Wang et al., 2014), we used The Cancer Genome Atlas (TCGA; cbioportal.org) database to explore known mutations in core clock genes of human GBM tumors (Brennan et al., 2013). Twenty-six missense, five nonsense and one in-frame deletion were identified in 16 genes critical for circadian timing (*CLOCK, ARNTL, ARNTL2, NPAS2, CRY1, CRY2, PER1, PER2, PER3, CSNK1D, CSNK1E, RORA, RORB, RORC, NR1D1 AND NR1D2*) in 1390 GBM samples (Figure 2). Thus, approximately 2% of GBM cases had mutations in at least one of the core clock genes. We then sequenced the DNA of our 5 GBM cell lines and found only one line (B18) carried one of the known clock gene mutations (PER1S784F). This line, however, possessed strong circadian rhythms. We conclude that human GBM cells are intrinsically circadian and mutations that affect their circadian timing are rare.

Murine mes-GBM astrocytes exhibit circadian rhythms in TMZ response

To further test the role of circadian timing in GBM biology, we generated a mouse model of GBM. Mouse cortical astrocytes rendered null for neurofibromin (Nf1) and P53 function (mes-GBM; (Sun et al., 2014)) were transduced with a real-time luciferase reporter for either *Bmal1* (*Bmal1-luc*) or *Period2* (*Per2-Luc*) (Liu et al., 2008; Ramanathan et al., 2012; Zhang et al., 2009). Cultured mes-GBM astrocytes expressed circadian, anti-phase rhythms for at least 4 days in *Per2-luc* and *Bmal1-luc* with periods of 23.6±3.2 h and 23.1±2.6 h, respectively (mean±SD; Figure 3A), consistent with their expression patterns in other cell types (Reppert and Weaver, 2002). Thus, endogenous circadian rhythms are conserved in human and mouse GBM cells.

To test whether the presence of circadian rhythms in gene expression affect GBM response to chemotherapy, we treated mes-GBM astrocytes with temozolomide (TMZ), the first-line chemotherapeutic for GBM, based on circadian time. Cells received either 1 mM TMZ or vehicle (DMSO) for 6 hours at 1 of 4 times phases of *Bmal1-luc* expression during the day after plating (Figure 3B). We counted viable cells by their ability to exclude trypan blue 72 hours after TMZ or DMSO addition. We defined growth inhibition as the number of TMZ-treated living cells divided by the number of DMSO-treated living cells at each treatment time (i.e. the death of all TMZ-treated cells would equal 100% growth inhibition). The greatest TMZ-induced growth inhibition occurred near the peak of *Bmal1-luc* expression

(Figure 3C). Thus, TMZ sensitivity of mes-GBM astrocytes varied with the phase of *Bmal1* expression.

To assess whether the rhythm in growth inhibition was due to rhythms in response to TMZ-induced DNA damage, we treated mes-GBM astrocytes with 1 mM TMZ or DMSO at 1 of 4 phases of *Bmal1-luc* expression during the day after plating (Figure 4A) and stained for phosphorylation of histone H2AX (γH2AX), an early step in DNA damage response (DDR) and a commonly used marker for DNA double-strand breaks (Bonner et al., 2008; Rogakou et al., 1999). We exposed cells to TMZ or vehicle for 6 hours, changed the media and fixed the cells 12 hours later. We quantified nuclear staining for γH2AX (p5Ser139; example staining in Figure S1) and calculated the fraction of γH2AX positive cells divided by the total DAPI-stained nuclei per field of view (Figure 4B). TMZ-induced γH2AX ($\gamma H2AX_{TMZ}$ - $\gamma H2AX_{DMSO}$) was maximal near the peak of *Bmal1-luc* expression (Figure 4C). There was a mean 2.85fold difference in TMZ response at the peak versus trough of *Bmal1-luc* expression across 3 independent experiments. These results demonstrate a time of day-dependent rhythm in the response of mes-GBM astrocytes to TMZ-induced DNA damage.

Daily rhythms in TMZ-induced γH2AX and growth inhibition led us to test whether there was a time of day-dependent rhythm in TMZ-induced apoptosis. Mes-GBM astrocytes stably expressing a luciferase reporter of caspase 3 and 7 activities (*Casp-luc*) (Galban et al., 2013) were treated with TMZ or DMSO for 6 hours at 1 of 4 phases of *Per2-luc* expression during the day after plating (Figure 5A). TMZ-induced activation of *Casp-luc* bioluminescence above the levels evoked by DMSO was calculated from measurements taken 48 hours after treatment. We expressed *Casp-luc* activation as fold change relative to the response at the *Per2-luc* falling phase (Figure 5B). There was a significant increase in *Casp-luc* bioluminescence when mes-GBM astrocytes were treated at the trough of Per2-luc, which in parallel dishes corresponded to the peak of *Bmal1*. Thus, TMZ-induced activation of apoptosis in the mes-GBM astrocytes had a circadian rhythm.

Circadian rhythms in TMZ-induced apoptosis are Bmal1-dependent

The correlation between high *Bmal1* expression and high TMZ sensitivity led us to hypothesize that increased TMZ sensitivity is *Bmal1*-dependent. We used CRISPR-Cas9 genome editing to disrupt the *Bmal1* gene (*Bmal1* KO) and abrogate BMAL1 protein expression (Figure S2). Loss of *Bmal1* abolished circadian rhythms in *Per2-luc* expression (Figure 5C). These data demonstrate disruption of the molecular clock in *Bmal1* KO mes-GBM astrocytes.

To determine the necessity of *Bmal1* expression to generate rhythms in TMZ-induced apoptosis, *Bmal1* KO mes-GBM astrocytes stably expressing *Casp-luc* were treated with TMZ at 1 of 4 times according to the *Per2-luc* rhythm of a WT culture (Figure 5A). Loss of *Bmal1* expression abolished the rhythm in TMZ-induced caspase activity (Figure 5D) and reduced the ratio of TMZ to vehicle *Casp-luc* activity to 15.3 to 42.3 percent of that observed in *Bmal1* WT across three independent experiments.

Discussion

Our study uniquely demonstrates endogenous circadian rhythms in human and murine GBM cells and their response to TMZ chemotherapy. We found cell-intrinsic daily rhythms in DNA repair, apoptosis and growth inhibition, with maximum TMZ sensitivity of all 3 measures occurring near the peak of *Bmal1* expression. Furthermore, our studies indicate that tailoring TMZ administration to the peak of *Bmal1* (trough of *Per2*) expression in tumor cells can enhance TMZ efficacy. Taken together, these data support a rhythm in tumor response to TMZ at the level of DNA repair and leads to a rhythm in programmed cell death.

These experiments build upon research that has previously demonstrated regulation of the DDR by the circadian clock. Through direct protein-protein interactions, PER1 and PER3 enhance activation of cell cycle arrest in response to DNA double-strand breaks (Gery et al., 2006; Im et al., 2010). Our study is the first to test circadian regulation of the DDR in glioma cells. It will be important to determine if other cell types exhibit circadian rhythms in their repair of DNA damage.

The correlation between the peak of *Bmal1* expression and the greatest sensitivity to TMZ combined with the loss of circadian rhythm in TMZ-induced apoptosis in *Bmal1* KO mes-GBM astrocytes suggests an important role for BMAL1 in regulating response to DNA damage. These findings are consistent with the daily variations observed in colon cancer cell sensitivity to irinotecan, achieving peak sensitivity at the peak of *Bmal1* transcription (Dulong et al., 2015). Overexpression of *Bmal1* increased sensitivity of colon cancer cells to oxaliplatin (Zeng et al., 2014). *Bmal1* knockdown reduced etoposide-induced apoptosis of colon cancer cells (Zeng et al., 2010). These data are consistent with our findings that loss of *Bmal1* leads to ablation of the rhythm in caspase activation and a reduction in maximal TMZ efficacy. There are no data showing a direct interaction of BMAL1 with proteins involved in apoptosis, but there are reports of *Per2* overexpression altering expression of apoptotic genes (Hua et al., 2006). Thus, BMAL1 may act as an indirect regulator of apoptosis through its role as a transcriptional activator. Irinotecan is also reported to induce greater cytotoxicty at the peak of *Bmal1*, suggesting circadian regulation of a common DNA repair pathway in response to both of these chemotherapies.

Future studies should test whether Bmal1 is playing a direct or indirect role in regulating the DDR and gliomagenesis (Jiang et al., 2016). Identifying rhythmic transcripts of DDR-related genes that lose rhythmicity in *Bmal1* KO cells would provide a list of candidate regulators. In contrast, identifying DDR-related proteins in complex with Bmal1 would suggest a more direct role. At present, the *Bmal1-luc* and *Per2-luc* reporters serve as useful tools to help us understand circadian clock regulation within tumor cells and to guide the search for the mechanism of circadian regulation of the tumor cell-intrinsic response to TMZ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the Children's Discovery Institute (JBR/EDH) and NIGMS 9687304 (EDH).

References

Barone A, Sengupta R, Warrington NM, Smith E, Wen PY, Brekken RA, Romagnoli B, Douglas G, Chevalier E, Bauer MP, et al. (2014). Combined VEGF and CXCR4 antagonism targets the GBM stem cell population and synergistically improves survival in an intracranial mouse model of glioblastoma. Oncotarget 5, 9811–9822. [PubMed: 25238146]

Beale P, Judson I, Moore S, Statkevich P, Marco A, Cutler DL, Reidenberg P, and Brada M (1999). Effect of gastric pH on the relative oral bioavailability and pharmacokinetics of temozolomide. Cancer Chemother Pharmacol 44, 389–394. [PubMed: 10501912]

Beaule C, Granados-Fuentes D, Marpegan L, and Herzog ED (2011). In vitro circadian rhythms: imaging and electrophysiology. Essays Biochem 49, 103–117. [PubMed: 21819387]

Bonner WM, Redon CE, Dickey JS, Nakamura AJ, Sedelnikova OA, Solier S, and Pommier Y (2008). GammaH2AX and cancer. Nat Rev Cancer 8, 957–967. [PubMed: 19005492]

Brennan CW, Verhaak RG, McKenna A, Campos B, Noushmehr H, Salama SR, Zheng S, Chakravarty D, Sanborn JZ, Berman SH, et al. (2013). The somatic genomic landscape of glioblastoma. Cell 155, 462–477. [PubMed: 24120142]

Buhr ED, Yoo SH, and Takahashi JS (2010). Temperature as a universal resetting cue for mammalian circadian oscillators. Science 330, 379–385. [PubMed: 20947768]

Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, and Bradfield CA (2000). Mop3 is an essential component of the master circadian pacemaker in mammals. Cell 103, 1009–1017. [PubMed: 11163178]

Dulong S, Ballesta A, Okyar A, and Levi F (2015). Identification of Circadian Determinants of Cancer Chronotherapy through In Vitro Chronopharmacology and Mathematical Modeling. Mol Cancer Ther 14, 2154–2164. [PubMed: 26141947]

Edelstein AD, Tsuchida MA, Amodaj N, Pinkard H, Vale RD, and Stuurman N (2014). Advanced methods of microscope control using muManager software. J Biol Methods 1.

Galban S, Jeon YH, Bowman BM, Stevenson J, Sebolt KA, Sharkey LM, Lafferty M, Hoff BA, Butler BL, Wigdal SS, et al. (2013). Imaging proteolytic activity in live cells and animal models. PLoS One 8, e66248. [PubMed: 23776643]

Gery S, Komatsu N, Baldjyan L, Yu A, Koo D, and Koeffler HP (2006). The circadian gene per1 plays an important role in cell growth and DNA damage control in human cancer cells. Mol Cell 22, 375–382. [PubMed: 16678109]

Hua H, Wang Y, Wan C, Liu Y, Zhu B, Yang C, Wang X, Wang Z, Cornelissen-Guillaume G, and Halberg F (2006). Circadian gene mPer2 overexpression induces cancer cell apoptosis. Cancer Sci 97, 589–596. [PubMed: 16827798]

Im JS, Jung BH, Kim SE, Lee KH, and Lee JK (2010). Per3, a circadian gene, is required for Chk2 activation in human cells. FEBS Lett 584, 4731–4734. [PubMed: 21070773]

Jiang W, Zhao S, Jiang X, Zhang E, Hu G, Hu B, Zheng P, Xiao J, Lu Z, Lu Y, et al. (2016). The circadian clock gene Bmal1 acts as a potential anti-oncogene in pancreatic cancer by activating the p53 tumor suppressor pathway. Cancer Lett 371, 314–325. [PubMed: 26683776]

Levi F, Giacchetti S, Adam R, Zidani R, Metzger G, and Misset JL (1995). Chronomodulation of chemotherapy against metastatic colorectal cancer. International Organization for Cancer Chronotherapy. Eur J Cancer 31A, 1264–1270. [PubMed: 7577034]

Liu AC, Tran HG, Zhang EE, Priest AA, Welsh DK, and Kay SA (2008). Redundant function of REV-ERBalpha and beta and non-essential role for Bmal1 cycling in transcriptional regulation of intracellular circadian rhythms. PLoS Genet 4, e1000023. [PubMed: 18454201]

Marpegan L, Krall TJ, and Herzog ED (2009). Vasoactive intestinal polypeptide entrains circadian rhythms in astrocytes. J Biol Rhythms 24, 135–143. [PubMed: 19346450]

Nakajima Y, Ikeda M, Kimura T, Honma S, Ohmiya Y, and Honma K (2004). Bidirectional role of orphan nuclear receptor RORalpha in clock gene transcriptions demonstrated by a novel reporter assay system. FEBS Lett 565, 122–126. [PubMed: 15135064]

Newlands ES, Blackledge GR, Slack JA, Rustin GJ, Smith DB, Stuart NS, Quarterman CP, Hoffman R, Stevens MF, Brampton MH, and et al. (1992). Phase I trial of temozolomide (CCRG 81045: M&B 39831: NSC 362856). Br J Cancer 65, 287–291. [PubMed: 1739631]

Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, and Schibler U (2002). The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 110, 251–260. [PubMed: 12150932]

Prolo LM, Takahashi JS, and Herzog ED (2005). Circadian rhythm generation and entrainment in astrocytes. J Neurosci 25, 404–408. [PubMed: 15647483]

Ramanathan C, Khan SK, Kathale ND, Xu H, and Liu AC (2012). Monitoring cell-autonomous circadian clock rhythms of gene expression using luciferase bioluminescence reporters. J Vis Exp.

Reppert SM, and Weaver DR (2002). Coordination of circadian timing in mammals. Nature 418, 935–941. [PubMed: 12198538]

Rogakou EP, Boon C, Redon C, and Bonner WM (1999). Megabase chromatin domains involved in DNA double-strand breaks in vivo. J Cell Biol 146, 905–916. [PubMed: 10477747]

Sato TK, Panda S, Miraglia LJ, Reyes TM, Rudic RD, McNamara P, Naik KA, FitzGerald GA, Kay SA, and Hogenesch JB (2004). A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. Neuron 43, 527–537. [PubMed: 15312651]

Schmiegelow K, Glomstein A, Kristinsson J, Salmi T, Schroder H, and Bjork O (1997). Impact of morning versus evening schedule for oral methotrexate and 6-mercaptopurine on relapse risk for children with acute lymphoblastic leukemia. Nordic Society for Pediatric Hematology and Oncology (NOPHO). J Pediatr Hematol Oncol 19, 102–109. [PubMed: 9149738]

Stevens MF, Hickman JA, Langdon SP, Chubb D, Vickers L, Stone R, Baig G, Goddard C, Gibson NW, Slack JA, and et al. (1987). Antitumor activity and pharmacokinetics in mice of 8-carbamoyl-3-methyl-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (CCRG 81045; M & B 39831), a novel drug with potential as an alternative to dacarbazine. Cancer Res 47, 5846–5852. [PubMed: 3664486]

Stevens RG (2005). Circadian disruption and breast cancer: from melatonin to clock genes. Epidemiology 16, 254–258. [PubMed: 15703542]

Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, Ludwin SK, Allgeier A, Fisher B, Belanger K, et al. (2009). Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol 10, 459–466. [PubMed: 19269895]

Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, et al. (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352, 987–996. [PubMed: 15758009]

Sun T, Warrington NM, Luo J, Brooks MD, Dahiya S, Snyder SC, Sengupta R, and Rubin JB (2014). Sexually dimorphic RB inactivation underlies mesenchymal glioblastoma prevalence in males. J Clin Invest 124, 4123–4133. [PubMed: 25083989]

Vitaterna MH, Takahashi JS, and Turek FW (2001). Overview of circadian rhythms. Alcohol Res Health 25, 85–93. [PubMed: 11584554]

Wang F, Luo Y, Li C, and Chen L (2014). Correlation between deregulated expression of PER2 gene and degree of glioma malignancy. Tumori 100, e266–272. [PubMed: 25688509]

Warrington NM, Woerner BM, Daginakatte GC, Dasgupta B, Perry A, Gutmann DH, and Rubin JB (2007). Spatiotemporal differences in CXCL12 expression and cyclic AMP underlie the unique pattern of optic glioma growth in neurofibromatosis type 1. Cancer Res 67, 8588–8595. [PubMed: 17875698]

Zeng ZL, Luo HY, Yang J, Wu WJ, Chen DL, Huang P, and Xu RH (2014). Overexpression of the circadian clock gene Bmal1 increases sensitivity to oxaliplatin in colorectal cancer. Clin Cancer Res 20, 1042–1052. [PubMed: 24277452]

Zeng ZL, Wu MW, Sun J, Sun YL, Cai YC, Huang YJ, and Xian LJ (2010). Effects of the biological clock gene Bmal1 on tumour growth and anti-cancer drug activity. J Biochem 148, 319–326. [PubMed: 20576619]

Zhang EE, Liu AC, Hirota T, Miraglia LJ, Welch G, Pongsawakul PY, Liu X, Atwood A, Huss JW, 3rd, Janes J, et al. (2009). A genome-wide RNAi screen for modifiers of the circadian clock in human cells. Cell 139, 199–210. [PubMed: 19765810]

Zhang R, Lahens NF, Ballance HI, Hughes ME, and Hogenesch JB (2014). A circadian gene expression atlas in mammals: implications for biology and medicine. Proc Natl Acad Sci U S A 111, 16219–16224. [PubMed: 25349387]

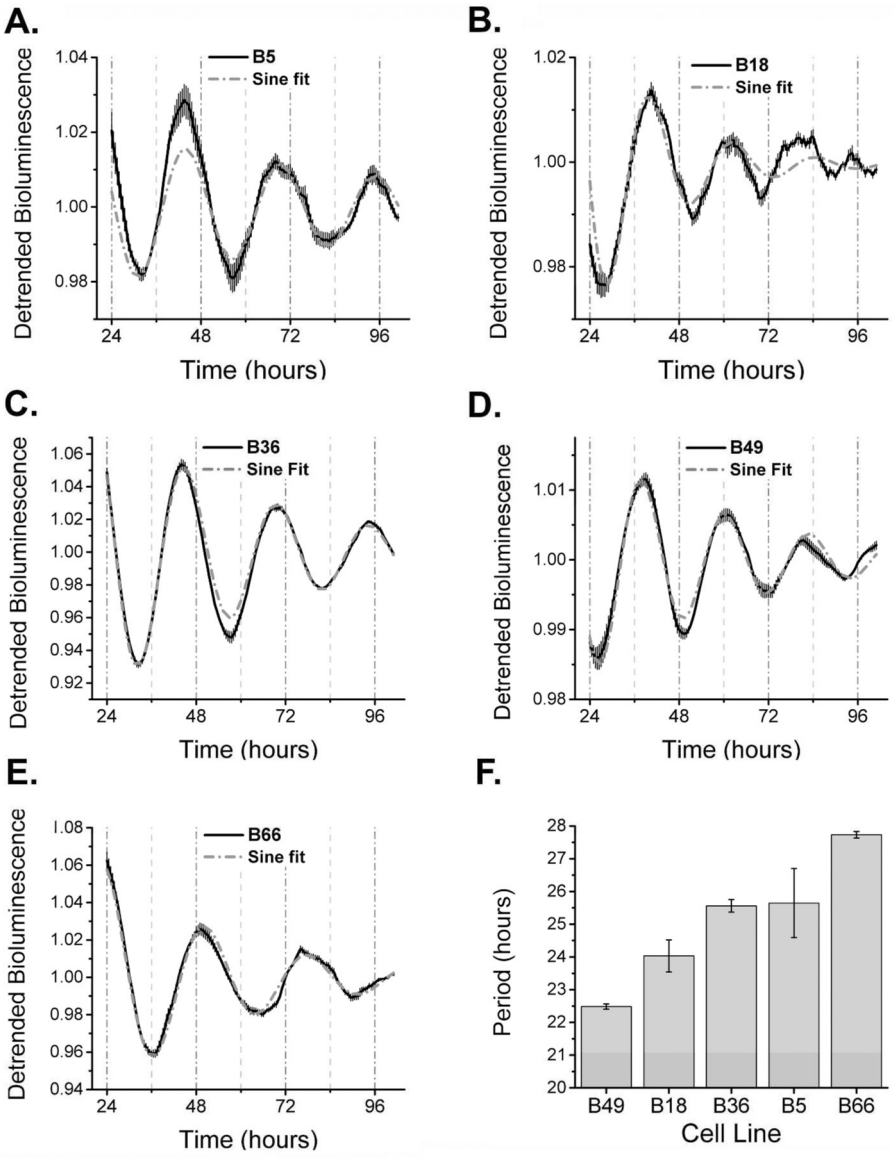
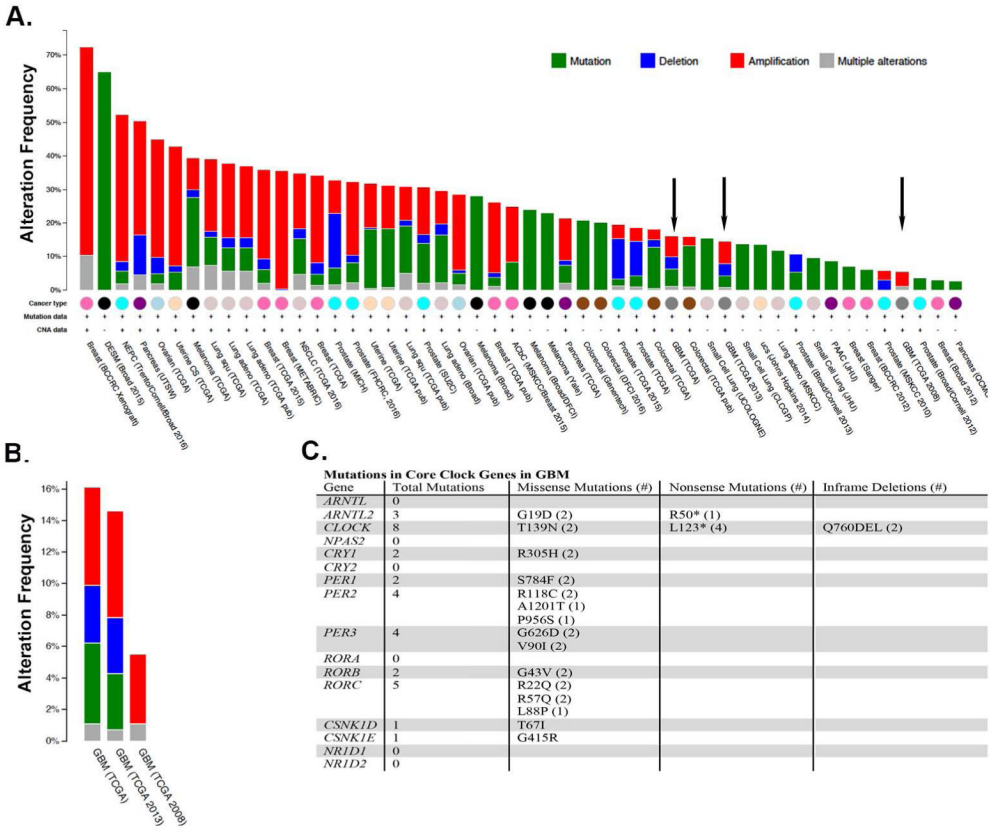


Figure 1: Human GBM cells are circadian.

(A-E) Representative bioluminescence traces of *Bmal1-luc* expression in the five human GBM cell lines (B05, B18, B36, B49, and B66). Each trace shows the mean (solid line) and SEM (grey error bars) of four replicate cultures fitted by a sine function (dashed line). Note that all cultures expressed intrinsic daily rhythms in *Bmal1-luc*. (**F**) Circadian period of the 5 GBM lines (Mean ± SD, n=4 independent platings).



Mutations in Core Clock Genes in GBM

Gene	Total Mutations	Missense Mutations (#)	Nonsense Mutations (#)	Inframe Deletions (#)
ARNTL	0			
ARNTL2	3	G19D (2)	R50* (1)	
CLOCK	8	T139N (2)	L123* (4)	Q760DEL (2)
NPAS2	0			
CRY1	2	R305H (2)		
CRY2	0			
PER1	2	S784F (2)		
PER2	4	R118C (2) A1201T (1) P956S (1)		
PER3	4	G626D (2) V90I (2)		
RORA	0			
RORB	2	G43V (2)		
RORC	5	R22Q (2) R57Q (2) L88P (1)		
CSNK1D	1	T67I		
CSNK1E	1	G415R		
NR1D1	0			
NR1D2	0			

Figure 2. Genetic alterations in circadian clock genes in cancer.

A) TCGA-based analysis revealed the frequency of amplifications (red), deletions (blue), mutations (green) or multiple alterations (gray) among 16 clock genes in different cancers. Arrows indicate evaluations of genetic alterations from 3 independent GBM tumor datasets. **B and C)** 32 mutations were identified in 16 core clock genes in three TCGA datasets comprised of 1390 GBM samples.

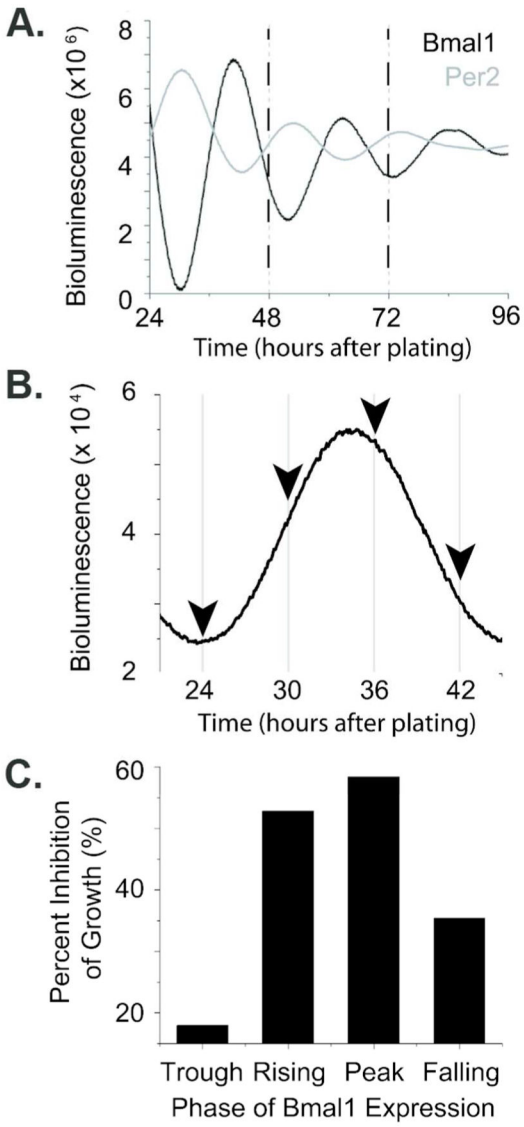


Figure 3. Mes-GBM astrocytes have rhythmic sensitivity to temozolomide *in vitro*.

A) Mes-GBM astrocytes express *Bmal1-luc* (black) and *Per2-luc* (gray) in anti-phase, with rhythmic periods of 25.3 h and 22.2 h, respectively, in these representative traces. **B**) A representative trace shows how mes-GBM astrocytes were treated with TMZ or DMSO at 1 of 4 times (arrows) in their daily *Bmal1* expression. **C**) TMZ-induced growth inhibition varied with time of treatment (1 representative experiment shown), peaking near the peak of *Bmal1-luc* expression in 3 independent biological replicates.

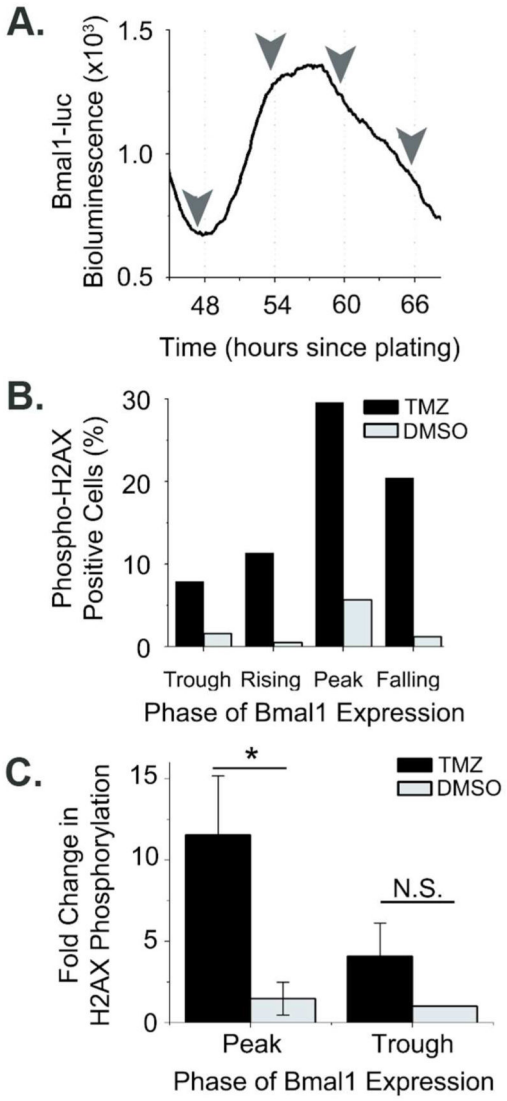


Figure 4. Phosphorylation of histone H2AX varies with time of treatment *in vitro*.

A) *Bmal1-luc* reporter mes-GBM cells showed oscillation of bioluminescence over time (one representative experiment, n=3). Arrows indicate times of TMZ or DMSO treatment for different mes-GBM cultures. **B**) Percent of phosphor-H2AX (γH2AX) positive cells varied with time of 1mM TMZ treatment (one representative experiment, n =3). **C**) Relative to γH2AX staining of mes-GBM astrocytes treated at the trough of *Bmal1* expression, TMZ induced a response at the peak, but not the trough, of *Bmal1* expression (Two-way ANOVA, Tukey's multiple comparisons, * = $p<0.05$, n= 3).

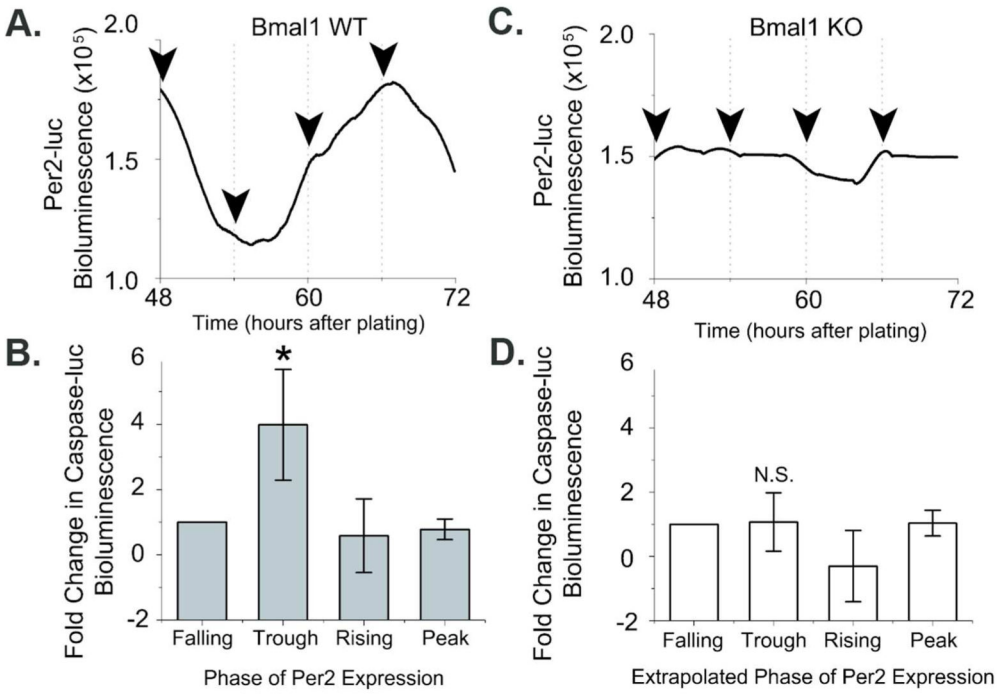


Figure 5. Rhythmic *Per2-luc* expression and activation of apoptosis depend on Bmal1 *in vitro*.
A) A representative culture of mes-GBM astrocytes showing circadian *Per2-luc bioluminescence*. **B)** TMZ-induced activation of a bioluminescent caspase reporter was highest when delivered at the trough of *Per2-luc* (i.e. peak of *Bmal1-luc*) (Kruskal-Wallis test, and Dunn's multiple comparisons test, $p<0.05$). CRISPR-mediated loss of *Bmal1* resulted in arrhythmic *Per2-luc* expression in mes-GBM cells. **D)** Caspase activation did not depend on the time of TMZ application in *Bmal1* KO mes-GBM astrocytes (One-way ANOVA, Dunn's multiple comparisons test, $p>0.05$).