ANALYSIS OF CIRCADIAN PROPERTIES AND CLOCK REGULATION OF GLIOMA AND BREAST CANCER STEM CELLS.

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Increased cancer risk is linked to disruption of circadian rhythms. Cancer stem cells (CSCs) are a known cause of cancer aggressiveness, but their circadian properties have not been described. In this study we describe the circadian properties of C6 rat glioma tumorspheres and MCF-7 human breast CSCs. We discovered circadian rhythms in gene expression within C6 glioma tumorspheres enriched in CSCs and found that the circadian clock is particularly robust in medium lacking any growth factors. A method is introduced for identifying individual CSCs in culture for single-cell analysis. CSCs in monolayer, attached cell cultures failed to show a circadian rhythm in nuclear localization of mPER2 protein, suggesting that cell interactions or the tumor-like microenvironment within tumorspheres enable circadian timing. The MCF-7 cancer cell line, derived from an epithelial breast tumor, has been widely studied because of its aggressiveness and high percentage of CSCs. Although several cancer cell lines have distinct circadian rhythms in gene expression, the reason why many other lines apparently lack circadian clocks remains unclear. Similarly, circadian rhythms of cells within tumors are also often poorly organized or absent. Considering the cell heterogeneity of cancer cell lines, including CSCs within these lines, it seemed likely that some of the cells could retain a functional circadian clock. To test this idea, we probed the circadian properties of MCF-7 cultures with a reporter gene that expresses a functional mPER2 protein fused with firefly luciferase under the control of the mouse Per2 promoter. MCF-7 cells grew as small clusters in medium containing fetal bovine serum or one containing growth factors stimulating CSC proliferation. The percentage of
clusters expressing the \textit{mPer2} gene was surprisingly high, and at least 60\% of these expressed a circadian rhythm. Reporter gene expression and \textit{Per2} mRNA were elevated in response to growth factors that prevent CSC differentiation. Despite previous reports suggesting otherwise, these results indicate that circadian clocks could have a functional role in MCF-7 breast cancer cells and that these clocks were previously undetected. Alternatively, the mouse \textit{mPer2} transfection may have rescued MCF-7 cells from an arrhythmic state. The presence of circadian rhythms in C6 and MCF-7 cultures enriched in CSCs suggests that there may be links between circadian clock genes and genes that inhibit CSC differentiation. These interactions should be tested further and might be used to develop novel therapeutic approaches for suppressing tumor growth by targeting CSCs.
I dedicate my PhD research to all my teachers and mentors, who have worked hard to transform me into a responsible and ethical scientist and a thoughtful human being.
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CHAPTER I: INTRODUCTION

Cancer

Cancer is not a new disease and afflicts people throughout the world. The word cancer was coined by the Greek physician Hippocrates (460-370 BC), also known as the “Father of Medicine”, but he was not the first to discover this disease. Some of the earliest evidence of bone cancer has been discovered in human fossils and it is explicitly discussed in ancient manuscripts dating back to 1600 B.C. However, in terms of treatment the manuscripts have only one thing to say: “There is no treatment.” According to inscriptions, surface tumors were removed surgically in a manner similar to how they are removed today.

Advanced research has identified important molecular targets within specific cancers, and drugs are being manufactured to treat many forms of cancer. Although chemotherapy has led to an increase in survival rates of individuals with certain types of cancers such as lung, cancers in brain, breast and pancreas still remain some of the most aggressive (Fig. 1). So the question arises: Why do many cancers remain incurable? The answer lies in the multiple properties of cancer. One of the known problems is that chemotherapeutic drugs used for cancer treatment are like double-edged swords, meaning they not only attack cancer cells but also normal cells of the body, thus leading to adverse side effects. More problematic and difficult to overcome is the problem of tumor recurrence and its two main characteristics: 1) A very small population of cancer cells are resistant to chemotherapy because they express a protein pumps in their membrane known as ABC protein (ATP-binding cassette protein). These proteins are able efflux cytotoxic chemicals from the cell including several known chemotherapeutic drugs. ABC protein isotypes comprise a huge family of proteins of which ABCG2 (also known as MDR1- multi drug
resistance protein 1) is well researched from the perspective of drug resistance particularly in glioma and breast cancer cells. 2) Some cancerous cells have an upregulated DNA damage repair system enabling them to repair DNA damage caused by chemotherapeutic drugs. For example, alkylating agents used for chemotherapy, such as temozolamide (TMZ) can methylate the 6th carbon of guanine residues in DNA, causing DNA polymerase to incorrectly add the thymine residue instead of cytosine to the replicating strand. A DNA mismatch repair protein identifies the mistake and in an attempt to correct it initiates a futile repair cycle eventually leading to cell death. However, some cancerous cells have MGMT (methyl guanine methyl transferase) overexpression allowing the cell to remove the methyl group thereby restoring the replication process and allowing cells to survive effects of TMZ. Cancerous cells with either of the two capabilities above, along with other properties not discussed here, are thought to exist in a small proportion of cells in solid tumors. Because they survive drug treatment these cells can repopulate to form a new tumor at the same site or in a different organ following metastasis. This resistant population has been termed the “cancer stem cells” (CSCs). CSCs not only survive the effects of most chemotherapeutic drugs but also renew themselves and differentiate into other cancer cells, thereby forming most of the tumor.
Figure 1: 2014 Estimated cancer case statistics. A) New case and death estimated for the year 2014. Breast cancer cases will be the most common ones to be reported. B) Five-year survival rates based on previous reports and treated cases (%). The values represent average for each period of surveillance. †= survival rate are significantly different between 2003-2009 and 1975-77 estimates. Source: American Cancer Society, Surveillance Research 2014.
Cancer Stem Cells

During development, organ-specific stem cells differentiate into organ-specific cell types. The distinction between embryonic stem cells and tissue-specific stem cells lies in the fact that the differentiation of organ-specific stem cells is largely restricted to cell types found within a particular organ. Based on this observation, a hypothesis developed recently has been called the “CSC hypothesis”. It states that the cellular origin of tumors is tissue-specific and tumors are driven by cellular components that display “stem cell properties”. The CSC concept by definition is actually not a new; the idea of cancer developing from a rare cell subpopulation in tissue is nearly 150 years old [1], [2]. Since then, several research articles have proposed that cancer originates from transformation of tissue-specific stem cells into CSCs [3], [2],[4]. The concept that tumors contain cell populations with stem cell properties was also suggested by in vitro studies, “clonogenic assays”, that showed subpopulations of tumor cells have an increased proliferative capacity in colony formation assays using cells isolated from tumor specimens [3].

Based on these observations CSC models were put forward to provide new approaches for treating cancer. In these models, a normal stem cell may accumulate mutations over time and transform into a cancer stem cell that will self-renew its own population at a slow rate and also give rise to progenitor or precursor cancer stem cells via asymmetric cell division. The progenitor cell can further differentiate to give rise to different cell types to form the tumor. Alternatively, a normal stem cell divides asymmetrically and produces a progenitor cell that accumulates mutations and transforms into a cancer stem cell, thus self-renewing its own population and differentiating into different tumor cell types.
The CSC hypothesis became more complex when it was shown that normal somatic cells can be reprogrammed to form stem cells by using a cocktail of transcription factors that are present only in stem cell populations [5]. Based on this observation it is hypothesized that normal somatic cells that accumulate mutations have the capability to de-differentiate into stem cell types and thus give rise to an entire tumor consisting of a heterogenic population of cells. This de-differentiation process can occur inside the tumor where properties of the cell microenvironment, for example hypoxia, favor expression of stem cell-specific genes [6, 7].

Cancer Stem Cell Models

Various cellular models have been proposed to explain the presence of stem-like cells in tumors and cancer cell lines. Although there appears to be great interest in studying CSCs in medical fields today, there are also controversies about models that attempt to describe the role of CSCs in primary tumors, as will be discussed in Chapter 6. According to one model, subpopulations of CSCs possess the capabilities of self-renewal, clonal sphere formation, and in vivo tumor formation, along with an ability to form progeny with a more restricted fate. This scenario includes a hierarchical lineage system in which the primary therapeutic cell target is the CSC itself (Fig. 2).

In contrast, the clonal evolution model includes no lineage hierarchy, and multiple cell populations result from various genetic mutations [8]. There is no cell hierarchy, because most of these cell subtypes self-renew and are capable of tumor formation. Therefore, all of the cells are targets of therapeutic intervention. In this complex system both genetic and epigenetic changes might occur within a single tumor, resulting in a multifaceted cell system where several tumor-initiating cell types may coexist. While genetic mutations may produce new tumor cell
populations, epigenetic changes might enable cells to produce progeny with a more or less restricted fate and also to temporarily adopt different states characterized by therapy resistance and expression of different cell markers. Another important feature of the clonal evolution model is that the individual cell populations interact. Whereas in this model all potential tumor forming cells postulated have to be targeted for successful therapy, the interruption of cell-cell and cell-niche interactions may also weaken the tumor system as a whole and provide benefits to patients.

A more modified CSC model is now gaining popularity among cancer investigators. The modified model takes both the hierarchical and clonal evolution models into consideration. It suggests that a cancer stem cell (CSC1) follows hierarchical model to self-renew and gives rise to the differentiated population. During the course of tumor progression the cancer stem cell may change by following a clonal evolution path to give rise to a new cancer stem cell (CSC2) (Fig. 3). The new CSC will also follow the hierarchical model to self-renew and propagate. Whether this model is true or not still needs to be validated. However, it explains the heterogeneity of the cancer stem cell markers observed in tumor tissue and cultured tumorospheres, which the hierarchical and clonal models substantially fail to explain.
**Figure 2: Cancer stem cell models.** In the linear hierarchy model of CSC formation, the transformation events that drive tumorigenesis occur in a stem (yellow) or progenitor cells that then give rise to more differentiated progeny as the tumor develops. These differentiated progeny have reduced tumor-forming potential. In the second model, cancer stem cells evolve, perhaps via induction of endothelial-mesenchymal transition (EMT), either as part of disease progression or in response to selective pressures within the tumor microenvironment [9].
Figure 3: Modified CSC model. Both Hierarchical and Clonal Evolution models of tumor maintenance may underlie tumorigenesis. Initially, tumor growth may be driven by a specific CSC (CSC1, red). With tumor progression, another distinctly different CSC (CSC2, blue) may arise due to clonal evolution of CSC1. This cell may result from the acquisition of an additional mutation or epigenetic modification. The more aggressive CSC2 becomes dominant and drives tumor formation. Both CSC1 and CSC2-type cells continue to propagate and self-renew following a hierarchical model.
Glioma Cancer Stem Cells

Glioma cancer can be classified into various types based on the severity of the tumor. Glioblastoma multiforme (GBM), a Grade IV brain tumor according to the World Health Organization (WHO) classification, is the most common form of primary brain tumor in the central nervous system (CNS), and its aggressive nature and evasiveness to treatments make it one of the most lethal cancers [10]. Current treatments for GBM, also called glioblastoma, range from common chemotherapeutic agents such as temozolomide (TMZ), paired with radiotherapy, to more recent anti-angiogenic agents and immunotherapeutic treatments [11, 12]. As with many cancers, however, anti-cancer therapeutic agents have not increased median survival of glioblastoma patients significantly over the past 10 years. The reason for the failure of treating glioma effectively and the recurrence of tumors has been largely attributed to a specific sub-population of the tumor, the glioma cancer stem cells (GSCs). There is obvious cellular heterogeneity within each glioblastoma tumor derived from patients. The cell population includes self-renewing, multi-potent cells (the GSCs) and more differentiated cells (non-GSCs) [13].

GSCs often have elevated expression of ABC pumps, providing resistance to chemotherapy and distinguishing them from other glioma cells [14, 15]. These “side population” cells can be identified by their negative nuclear staining with the fluorescent dye Hoechst 33342, which is exported by ABC pumps [16]. Another distinctive feature of GSCs is their ability to form tumorspheres in cell cultures, which are aggregates of cells with a tendency to detach from the cell monolayer, a property attributed to aggressive cancer cells. Tumorspheres contain cells capable of forming tumors and possibly additional GSCs that are differentiating into various cell types [17, 18].
Gene expression required for stem cell renewal and differentiation overlaps considerably between normal neural stem and progenitor cells and glioma stem cells. When glioma cells are in the presence of specific growth factors they are known to overexpress several genes, most notably Nestin, Bmi-1, CD133 and Musashi-1 (Msi-1). Of these, Bmi-1 is of particular interest because it is not only activated by the sonic hedgehog pathway but it is also found to be redistributed on the chromatin upon gamma radiation treatments [19].

Breast Cancer Stem Cells

Breast cancer is the major cause of cancer-related death among women worldwide, and in USA alone there are more than 40,000 breast cancer fatalities annually. In the last decade, early diagnosis of breast cancers in women has improved the prognosis. However, patients diagnosed with advanced-stage breast cancer continue to have poor survival rates. The difficulty in treating advanced breast cancers is mostly attributed to the existence of breast CSCs [20]. Where breast CSCs originate is not clearly understood. However, research in the past decade has identified several mechanisms by which breast CSCs initiate. One of the most accepted hypotheses is that CSCs originate from mammary multipotent stem cells as a consequence of genetic defects caused by damaging agents from the environment that affect pathways governing self-renewal and differentiation (Fig. 4) [21]. The adult human mammary gland is composed of a series of branched ducts and lobulo-alveolar units embedded in fatty tissue and is composed of three cell lineages: (i) myoepithelial cells, which form the basal layer of ducts and alveoli; (ii) ductal epithelial cells and (iii) alveolar epithelial cells, which line the alveoli and are capable of milk protein production [22], [23], [24]. Each of these special cell types in the mammary gland can be identified using cell type specific markers such as epithelial specific antigen (ESA) that is present on all epithelial breast cells. In the human breast epithelium, a stem cell hierarchy might
exist that is important for normal mammary organogenesis and function. For example the breasts undergo dramatic transformation both physically and physiologically during pregnancy, and a smooth transition through this transformation requires stem cells that can produce the relevant cell types.
Adapted from: Shiptsin, M and Polyak, K. Laboratory investigation, 2008, 88:5(459-462)

**Figure 4: Breast cancer stem cell model.** The normal breast stem or progenitor cells with bipotential capability and self-renewing capacity may transform into carcinogenic cells which then would undergo EMT transition, by mechanisms which are not well defined, and give rise to a breast tumor.
Mammalian Circadian Clock

A wide variety of physiological and behavioral rhythms are regulated by the circadian timing system [25, 26]. The mammalian circadian system organization exhibits a hierarchy of oscillators. The primary oscillator is the suprachiasmatic nucleus (SCN) of the anterior hypothalamus of the brain (Fig. 5). Even though the SCN does not drive the circadian rhythms in non-SCN cells, it appears to be necessary for synchronization of rhythms within tissues to distinct phases [27]. Additionally, circadian rhythms of behavior such as activity–rest cycles and physiological parameters such as body temperature are controlled by SCN rhythms, and SCN lesions eliminate the 24-hour timing of many behaviors. Interestingly, it was shown that the behavioral rhythms of an SCN-lesioned animal can be restored by transplantation of donor SCN into the third ventricle [28]. The SCN can synchronize peripheral oscillators by different modes, neural and hormonal, so that a coherent rhythm is orchestrated at the organismal level [29, 30].

A key characteristic of peripheral oscillators is their ability to respond to SCN-driven timing signals. In general, the SCN responds primarily to environmental light signals that entrain its clock to the local time of day, whereas peripheral clocks are thought to entrain to a complex and redundant combination of rhythmic neural, hormonal, and activity-dependent signals such as body temperature and the timing of food intake. In the entrained state, circadian clocks match their period to the period of the rhythmic entraining signal through shifts in the phase of the circadian pacemaker, the molecular timing mechanism. The endogenous period of circadian pacemakers typically differs from the 24-hour cycles of Earth and ranges from about 19 to 29 hours.
Figure 5: The mammalian circadian system. The hierarchical organization of the mammalian circadian timing system. Variety of signals from the SCN synchronize peripheral oscillators, such as direct neural signals through the autonomous nervous system, neuroendocrine signals such as glucocorticoids, and indirect signals such as circadian body temperature and food intake.
Peripheral Clocks

The clock mechanisms in the SCN and peripheral oscillators are known to be similar at the molecular level [31, 32], and both consist of a network of transcriptional–translational feedback loops (TTFL) that drive rhythmic, approximately 24-h, expression patterns of core clock components (Fig. 6). Core clock components are defined as genes whose protein products are necessary for the generation and regulation of circadian rhythms within individual cells throughout the organism. Furthermore, alterations of the levels of these proteins changes the phase of the circadian clock. The basic-helix-loop-helix (bHLH) transcription factors CLOCK and BMAL1 dimerize and form the primary loop as they activate transcription of the *Period* (*Per1, Per2 and Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) genes. The PER and CRY proteins dimerize and subsequently feedback at their own gene promoters to abrogate their transcription by directly inhibiting gene activation by the CLOCK:BMAL1 complex. A second feedback loop stabilizes the first loop and is composed of REV-ERBα and RORα, two retinoic acid-related orphan receptors, that repress and activate, respectively, transcription of *Bmal1* [33]. CLOCK expression, on the other hand, is constitutive and non-rhythmic in mammals.

Termination of the repression phase of PER and CRY is an essential step in setting up the TTFL. Termination of PER and CRY-induced repression and restart of a new cycle of transcription is brought about by enzymes casein kinase (CK)1ε and CK1δ. CK1ε/δ-mediated phosphorylation targets PER proteins for ubiquitination by βTrCP and degradation by the 26S proteasome. Unlike PER, CRY proteins are phosphorylated by a different set of kinase enzymes. AMPK1 and DYRK1A/GSK-3β that phosphorylate CRY1 and CRY2, respectively. Following phosphorylation CRY is polyubiquitinated by FBXL3 protein, thereby targeting it for
proteosomal degradation. The stability and degradation rates of PER and CRY is key to setting the circadian period of the clock.
Adapted from: Ethan D. Buhr and Joseph S. Takahashi, Handbook of experimental pharmacology, 2013; 217 (3-28)

**Figure 6: Schematic of the molecular circadian clock of mammals.** CLOCK/BMAL1 heterodimers (green and blue ovals) bind DNA of clock target genes at E-boxes or E'-boxes and initiate the transcription of their RNA. The resulting PER and CRY proteins (red and yellow ovals) dimerize in the cytoplasm and translocate to the nucleus where they inhibit CLOCK/BMAL1 proteins from initiating further transcription.
Clock-Controlled Genes

In addition to the core clock genes, hundreds or even thousands of genes are expressed with a circadian rhythm in various tissues, which are commonly referred to as clock-controlled genes (CCGs). Until the implementation of genome tools for analysis of rhythmic transcripts, the extent to which the mammalian transcriptome is circadian regulated was unknown. Based on several studies in the last decade we now know that between 2% to 10% of mammalian transcript in various tissues is under circadian control and these gene products are involved in diverse pathways depending on the tissue [34-38].

Circadian Rhythms and Cancer

It is well established that physiological, biochemical, and behavioral functions are regulated by circadian clocks in individual cells [39-41]. A major consequence of a modern lifestyle is occasional or frequent disruption of circadian rhythms. Disruption of the circadian timing system by environmental factors or sleep patterns is associated with higher cancer incidence and more malignancy [40, 42-44]. Core clock genes such as mPer2 that comprise the timing mechanism have been characterized as tumor suppressors and as mitotic regulators [45]. Circadian genes also have non-clock functions, which are important in regulation of cell cycle progression, DNA damage responses, and genomic stability. Therefore, clock and non-clock functions are possible contributors to the association between disruption of circadian rhythmicity and cancer.

Cancerous and non-cancerous glial cells have the ability to generate circadian rhythms. Glioma cell lines that are used in research on brain tumors display circadian rhythms in gene expression [46], although it is possible that only some of the various cell types within these lines
are competent circadian pacemaker cells capable of generating rhythms. Embryonic stem cells are reported to lack circadian rhythms, but the rhythm is apparent when the cells have differentiated [47]. The possibility of a functional circadian clock in cancer stem cells, and GSCs in particular, has not yet been examined.

Circadian rhythms are also present in primary astrocyte cultures [48]. Interestingly, glioma cancer cells isolated from patients and glioma cell lines are heterogeneous and contain cells that vary according to their state of differentiation. Although the circadian properties of glial cell lines have been examined in their entirety, the circadian properties of the cancer cell subtypes have not been characterized. It is speculated that circadian clock cells can influence cancer cell growth and cell differentiation; however, it is possible that only some of the cancer cell subtypes are competent circadian pacemaker cells.

The C6 glioma cell line was derived from a chemically-induced rat tumor [49] and consists of multiple types of glial cancer cells and neuron-like cells [50, 51]. The C6 line has been valuable for isolating and studying GSCs and also stem-like cancer cells that are considered to be progenitor cells [51]. Robust circadian rhythms in clock gene expression have been recorded from C6 cells transfected with reporter genes based on firefly luciferase bioluminescence and grown as monolayers [46].

One project described in this dissertation, identified and characterized a morphologically distinct H33-negative subpopulation within the C6 cell line and provided evidence that it differs from the remaining cells because it lacks a circadian rhythm in the nuclear localization of mPER2 while in monolayer cultures. In contrast, C6 tumorspheres enriched in GSCs did express circadian rhythms. The unique circadian properties of this stem-like subpopulation could
enable targeting of the cells with treatments to eliminate GSCs in glioma. Furthermore, by manipulating how their circadian genes are regulated it may be possible to drive GSCs to differentiate, thereby improving efficacy of existing cancer therapies.

A second project examined an important breast cancer cell line, MCF-7, known to contain a high percentage of stem cells. MCF-7 cells are derived from breast epithelial cancer cells and, like C6, are heterogenic. Published studies have reported a lack of circadian rhythms in MCF-7 cells growing in serum containing medium. However, circadian rhythms in MCF-7 tumorspheres have not been examined previously.
CHAPTER II: CIRCADIAN PROPERTIES OF CANCER STEM CELLS IN GLIOMA CELL CULTURES AND TUMORS PHERES

Introduction

Like normal cells, cancer cells contain molecular clocks that generate circadian rhythms in gene expression and metabolic activity [52-54]. The relationship between the circadian timing system and cancer is evident from studies linking disruption of circadian rhythms with higher cancer risk and greater malignancy [43, 44, 55-57]. In both cancer and normal cells, a rhythmic nuclear translocation of mPER2 and other critical clock proteins is an essential part of a clock timing mechanism based on transcriptional-translational feedback loops and rhythmic chromatin modification [58-60]. Key gene products serving in the circadian clock are important targets for manipulating cancer growth [61, 62].

Although many cancer cells are rhythmic, the circadian properties of tumors are more complex than those observed in most cancer cell lines. For example, expression of core circadian clock proteins that have been characterized as tumor suppressors and mitotic regulators [45, 61-64] are perturbed in tumors relative to non-cancerous tissue and cell lines [65-67]. The complexity of the tumor microenvironment and its cellular heterogeneity [68, 69] are possible causes of this disruption. Although several studies have examined circadian clocks of immortalized cells and cancer cell lines [52, 67, 70-72], the circadian properties of cancer stem cells (CSCs), a small but important tumor subpopulation, are largely unknown.

CSCs often have high expression of cell membrane drug transporters—e.g., ATP-binding cassette pumps (ABCG1 and ABCG2)—providing resistance to chemotherapy and distinguishing them from other cancer cells [18, 73, 74]. These “side population” (SP) cells can be identified by their negative nuclear staining with the fluorescent dye Hoechst 33342, which is
exported by the ABC pumps [16]. Even though CSCs are typically a small percentage of cancer cell lines, an ability to identify CSCs by their Hoechst 33342-negative (H33-) staining would enable examination of the cellular physiology of live CSCs easily and at high magnification. Once located, the CSCs of monolayers could be examined using numerous fluorescent probes. Pharmacological treatments and other modulators of cell activity could also be introduced easily into monolayers to test CSC properties.

Glioma tumorspheres are typically made from cancer cell lines or primary glioma by culturing the cells in serum-free medium containing epidermal growth factor (EGF), fibroblast growth factor-2 (FGF2), platelet-derived growth factor (PDGF), and leukocyte inhibitory factor (LIF) [75, 76]. Spheres have also been generated from monolayers of dissociated human glioma in the absence of these growth factors or serum (mitogen-free medium) [77]. Spheres composed of neural progenitor cells have been shown to express circadian rhythms [78]. Hematopoietic stem cells lack circadian rhythms in expression of all core clock genes except mPer2 [79]. Similarly, two studies have shown that embryonic stem cells lack circadian rhythms and that the rhythm appears as the cells differentiate or shortly thereafter [47, 78]. Similarly, undifferentiated cells of the testis [80-82] and thymus [83] lack autonomous circadian rhythms even though several circadian clock genes are expressed. Surprisingly, embryonic stem cell cultures show circadian rhythms in glucose utilization without detectable circadian rhythms in clock gene expression [84].

Because the possibility of an autonomous circadian clock within CSCs has not been examined in any species, we explored the circadian properties of CSCs in C6 rat glioma cells that express robust circadian rhythms in clock gene expression [46]. The C6 cell line was derived from a chemically-induced rat tumor [49] and can differentiate into cells with properties of
astrocytes, oligodendrocytes, and neurons [85-89]. In this study we used bioluminescence imaging (BLI) to describe for the first time circadian rhythms in C6 tumorspheres. We also characterized CSCs as a morphologically distinct H33- subpopulation within the C6 cell line in spheres and monolayers and provide evidence that they lack circadian rhythmicity when in monolayers but not in the tumorsphere microenvironment.

Materials and Methods

Cell culture, transfection, and reporter gene assay.

Rat C6 glioma cells obtained from American Type Cell Culture (catalog number CCL-107) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% FBS and with no pyruvate or phenol red and incubated at 37°C in 5% CO2. Cells were cotransfected with mPer2::mPer2:luc to produce a fusion protein containing mPER2 and firefly luciferase [47] and CMV::neo, following a standard transfection protocol (HilymaxH357-10, Dojindo). Other cells were transfected with a CMV::mPer2:GFP [90] construct that produces a fusion protein shown previously to undergo nuclear translocation. Transfected cells were selected with G418 (1 mg/ml) in DMEM and 10% FBS (v/v) for 7-10 days. Selected cells were grown in either high glucose DMEM without pyruvate and with 10% FBS added (serum medium, SM) or in a stem cell medium (SCM) containing 20 ng/ml EGF; 15 ng/ml bFGF2; and 15 ng/ml PDGF in the same DMEM and without serum. Tumorspheres generated in either medium were isolated for imaging or maintained in the same DMEM with no serum or exogenous growth factors (mitogen-free medium, MFM) for up to five weeks before imaging. Penicillin and streptomycin were added to all media.
Identifying C6 SP cells in monolayer cultures.

Cells were seeded (10^5 cells/dish) on 25-mm cover glasses placed in 35-mm tissue culture dishes and incubated in DMEM containing 10% FBS at 37°C with 5% CO2. The following day, cells were washed twice with a 10 mM HEPES-buffered, low-bicarbonate, phenol red-free and mitogen-free medium (HMFM) designed for use in room air and stained with Hoechst 33342 (10-20 ng/ml, Invitrogen) for 1 min. The cells were then washed with HMFM and then incubated in HSM, with FBS lowered to 5%, at 37°C without CO2 for 2 hrs before imaging. When comparing H33342-stained cells in SM and SCM the cells were treated with Hoechst two and four days after cell plating and fields of view at 20x magnification were selected at random for cell counts. An observer blind to the dish’s prior treatment method counted cells in 15 image frames from each treatment.

Fluorescence and bioluminescence imaging.

The mPer2::GFP cultures were incubated at 37°C and imaged at room temperature with a 12-bit interline-transfer, cooled-CCD camera (MicroMax, Princeton Instruments), a 20x objective lens, Axiophot fluorescence microscope (Zeiss), and MetaMorph software. GFP images were collected with 5-sec exposures and 488 nm excitation and 507 nm emission filters. Hoechst images were collected with 0.1-sec exposures and 352 nm excitation and 461 nm emission filters. Reference images were captured using differential interference contrast (DIC). All images were analyzed with ImageJ (NIH) and processed using Photoshop (Adobe).

The mPer2::mPer2::luc C6 tumorsphere cultures were moved to a temperature-controlled chamber at 37°C and imaged from above with a 16-bit back-thinned, back-illuminated cooled-CCD camera (CH360, Photometrics) and a 50-mm Nikkor f/1.2 lens (Nikon) combined with a close-up lens (+10 diopter). A temperature-controlled optical window, covered with a
transparent indium-tin-oxide layer, was used to prevent condensation and was sealed with sterile silicone vacuum grease to the culture dish. Bioluminescence images were captured with 2x2 pixel binning and 30-min exposures using V++ software (Digital Optics). Media used during imaging were 10 mM Hepes-buffered versions of SM (HSM), SCM (HSCM), or MFM (HMFM) with sodium bicarbonate levels lowered for use in room air, as in Hank’s Balanced Salt Solution.

MFM tumorspheres generation and maintenance.

The mPer2::mPer2::luc tumorspheres were generated by seeding cells at low density in SM. The medium was changed every 4 to 5 days and the cells were passaged when the culture plate became confluent. Once the tumorspheres were visible by eye they were isolated using a 200-µl broad-mouthed pipet tip and transferred to a sterile 15-ml tube (8 to 10 per tube) containing 1 ml MFM. Tumorspheres were allowed to settle to the bottom, after which medium was aspirated using a sterile 1-ml pipet. Spheres were then washed twice with 1 ml fresh MFM and transferred to 60-mm (2 to 5 spheres) or 100-mm (5 to 10 spheres) dishes containing MFM. This step ensured that the plates were not crowded with spheres and nutrients in the medium were not depleted. Spheres were incubated at 37°C with 5% CO2 and humidity for 2 or 4 weeks. At 3-5 days before imaging the 2-week or 4-week-old spheres were transferred to a 35-mm dish containing 3 ml HMFM. Immediately before imaging 6 µl of 100 mM luciferin in water was added, followed by gentle mixing, to bring the luciferin concentration to 200 µM. Following the addition of luciferin, spheres were imaged for 3 to 4 days without forskolin treatment. Next, the dish was moved to a sterile hood, medium was replaced with 20 µM forskolin in HMFM, and the spheres were incubated for 2 hrs at 37°C. Forskolin medium was replaced with 1 ml HMFM, which was then replaced with 3 ml HMFM containing 200 µM luciferin. BLI resumed and continued for another 3 to 4 days, providing a total of 6 to 8 days of imaging.
Confocal microscopy.

C6 tumorspheres were generated in SM or SCM and maintained in HMFM for confocal microscopy. Tumorspheres were stained with Hoechst 33342 as described above, washed with MFM, and incubated at 37°C for two hrs. Following incubation the spheres were fixed in tissue culture dishes with 100% methanol for 5 min. Spheres were washed and stained with propidium iodide (PI) diluted 1:3000 for 3-4 min and then mounted in 30% glycerol on a No. 1 cover glass. Confocal microscopy of spheres was performed with a Leica-TCS SP5 multi-photon laser scanning confocal microscope using 345 nm excitation/460 nm emission (Hoechst) and 536 nm excitation/617 nm emission (PI), a Z-axis step size of 2 µm, and a 20x objective.

Isolating spheres from C6-mPer2::mPer2:luc cultures.

C6 mPer2::luc spheres were isolated from tissue culture dishes manually using 1000 µl pipette tips or broad-mouthed 200 µl pipette tips and transferred to 35-mm dishes containing HSM medium. Dishes containing floating spheres were isolated by decanting the medium into a 35-mm dish followed by incubation at 37°C overnight after which medium was replaced with fresh HSM medium. Some tumorspheres were washed thoroughly in HMFM medium and transferred into HMFM medium and then maintained in HMFM for at least 2-3- weeks before being imaged.

Stem cell markers in spheres and monolayer cells.

Immunofluorescence staining was used to identify CD133 and GFAP-positive cells in isolated spheres and surrounding cell monolayer. Antibodies against CD133 (Miltenyi Biotech) phospho-H3S10 (Cell Signaling); nestin, beta III-tubulin and MAP2 (Aves Labs); musashi1 (Cell Signaling); olig2 (Phospho Solutions); and glial fibrillary protein (GFAP, Cell Signaling) were used along with Alexa545 and Alexa488-conjugated secondary antibody (Life Technologies).
Frozen sections of tumorspheres were prepared using a freezing microtome (Super Freeze, Fisher Scientific). Spheres were fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) and standard immunostaining was performed.

Data analysis.

The GFP signal in transfected C6 cells of monolayer cultures was measured by drawing a region-of-interest (ROI) over cytoplasmic regions and nuclei of fluorescence images. The nuclear/cytoplasmic ratio was calculated using the maximum intensity within each ROI after background fluorescence was subtracted. Bioluminescence images were analyzed by using the minimum signal of adjacent frames to create images free of cosmic ray-associated artifacts and then drawing a single ROI over each sphere at each time-series frame. Detrending the BLI data consisted of a 24-point running average subtraction, as described previously [91]. A five-point running average was then applied, and the time when peaks occurred was measured. Period estimates were taken as the average time between peaks during the first three circadian cycles. Period stability was determined as the average difference between the periods of the 1st and 2nd circadian cycles. Modulo 24 hrs of the time of the first peak of a circadian cycle from the start of the time series was used to calculate the phase of the oscillation. SM sphere forskolin treatments were applied immediately before imaging began, but forskolin was applied to SCM and MFM spheres after they had been imaged for 3-4 days, in which case a new time-series began immediately after the treatment and, because of their movement, additional spheres were imaged after the treatment. Bioluminescence and fluorescence images were analyzed with ImageJ (NIH) and OriginLab software. Using Oriana circular statistics software (Kovach Computing Services), a Rayleigh’s test for uniformity was applied to determine whether the rhythm phases were significantly clustered around a preferred phase. Other data set means were compared
statistically by one-way analysis of variance (ANOVA) and the Scheffe post-hoc test with a significance of p>0.05 or less.

**Results**

Hoechst-negative cells in monolayer cultures are a distinct stem-like subpopulation.

To identify H33- cells in C6 monolayers we used a dye concentration similar to that used for sorting cells by flow cytometry (4-7 µg/ml), but detected only H33+ cells. H33- cells were, however, readily identifiable in the cell monolayer cultures after staining with H33 at 5-20 ng/ml. (Fig. 7A), Because serum-free stem cell medium (SCM) containing FGF2, EGF, and PDGF should select for growth of CSCs, we determined the density of H33- and H33+ cells after 2 and 4 days in SCM or serum medium (SM) containing 10% FBS. We compared two cell cultures made from the same C6 cell dispersal plated at equal cell density into culture dishes containing either SM or SCM. Density of H33- cells was significantly higher in SCM than in SM at days 2 and 4 (Fig. 7B), but density of H33+ cells was not significantly different between SCM and SM at either time. The percentage of H33- cells was 17.38 ±1.739 and 16.24 ±1.274 in SCM at days 2 and 4, respectively, versus 6.09 ±0.88 and 5.05 ±0.748 in SM, indicating an approximately threefold increase within the first 48 hrs in SCM. We also measured the diameters of both cell types and found that H33- cells were significantly smaller than H33+ cells in SM and SCM (Fig. 7C-E).

C6 H33- cells showed rounded morphology rather than the polygonal shape typical of the H33+ cells. Because apoptotic cells will show dense positive staining with the Hoechst dye [92, 93], we conclude that H33- cells of the monolayers are not apoptotic cells. After immunolabeling two C6 cultures grown in SCM on a cover glass for 48 hrs, H33- cells showed
high expression of CSC markers CD133 and musashi1 (Fig. 11). No fluorescence was observed in cultures in which primary antibodies were omitted.
Figure 7: Proliferation and size of Hoechst-negative cells in stem cell medium. A: DIC reference image and cells with Hoechst-positive and negative nuclear staining. Bar=50 µm. Arrow: Hoechst-negative CSC. B: Density of Hoechst-negative and Hoechst-positive cells grown in serum medium (SM) or stem cell medium (SCM) for 2 (Black bars) or 4 (White bars) days. Hoechst-negative, but not Hoechst-positive cells, propagate significantly faster in SCM (*) than in SM (ANOVA, F=11.05, p<0.001) C: Diameters of Hoechst-negative (red) and Hoechst-positive (black) cells grown in serum medium (SM) for 1-3 days. Hoechst-positive cells are significantly larger than Hoechst-negative cells (mean=53.90 µm ±14.19 SD, n=715 versus 19.18 µm ±4.03 SD, n=141; t-test: t=28.18, p<0.001). D: Hoechst-positive cells are also significantly larger than Hoechst-negative cells in C6 cultures grown in stem cell medium (SCM) for 2 days (mean=23.3 µm ±6.4 SD, n=172 versus 8.0 µm ±2.7 SD, n=89, t=21.44, p<0.001) or 4 days (mean=28 µm ±10.6 SD, n=152 versus 8.5 µm ±3.7 SD, n=129, t=19.31, p<0.001) (E).
The Hoechst-negative subpopulation in cell monolayers does not exhibit a circadian rhythm.

Because other poorly differentiated cell types are reported to lack circadian rhythms, we used fluorescence imaging of clock protein localization within individual cells to evaluate which C6 cell types are rhythmic. C6 cells were transfected with a CMV::mPer2::GFP reporter gene [90] (Fig. 8A-D). Circadian rhythms in nuclear translocation of mPER2-LUC were predicted from previously demonstrated nuclear entry of this fusion protein via its interaction with CRY proteins that accumulate rhythmically in the nucleus [59, 90].

Fluorescence images were taken at 11 time points over 56 hrs from cultures given a 20 µM forskolin pulse to synchronize individual circadian clock cells. A new culture dish was used at each time point. We calculated the ratio of the GFP fluorescence intensity in the nucleus to that in the cytoplasm and observed that H33- cells did not show circadian rhythms (Fig. 8C, D). Both cell types displayed high (about twofold) mPER2-GFP accumulation in the nucleus immediately after the 2-hr forskolin pulse, whereas untreated cells showed both high and low nuclear signals in both cell types (Fig. 8A-D). Cells used in Figure 8C, D were also used for cell diameter measurements shown in Figure 7C.
**Figure 8: Circadian rhythms in mPER2 nuclear localization.** A: DIC with Hoechst. B: overlay of mPER2-GFP and Hoechst signals. High (black arrow) and low (Red arrow) nuclear localization of mPER2-GFP in Hoechst-positive cells. White arrow: Hoechst-negative putative CSC. C: Hoechst-positive (non-CSC) cells show circadian rhythms in mPER2-GFP nuclear localization (nuclear/cytoplasmic ratio). D: The Hoechst-negative cell population is not rhythmic. Day 0 begins at end of forskolin treatment used to synchronize cells.
Tumorspheres are enriched in Hoechst-negative cells and have functional circadian oscillators.

Because H33- cells did not display circadian rhythms in monolayer culture, we examined whether tumorspheres might be rhythmic because of the distinctly different microenvironment within tumorspheres. Spheres formed spontaneously in SM in normal plastic cell culture dishes without needing a low-attachment surface and grew in clusters that detached from the surface to become floating spheroid bodies. We performed Hoechst staining in the live spheres using the same procedure developed for monolayers and then stained with propidium iodide after fixation to identify cell nuclei. When stem cell properties of SM-grown spheres were compared with ones grown in SCM, the percentage of sphere volume occupied by H33- cells was very similar according to confocal microscopy measurements. With this approach about two-thirds of the cell population of the spheres consisted of H33- cells (SM: 66.5% ±3.25% and SCM: 56.5% ±5.1%, n=2 each. ±SD) (Fig. 9). Immunolabeling the spheres with an antibody against the mitotic marker H3-S10p indicated low ongoing cell division restricted to a few cells scattered throughout each sphere, as shown in composite confocal microscopy images of entire spheres (Fig. 9A, B). When SM-derived spheres were maintained in medium with no serum and no added growth factors (mitogen-free medium, MFM) for at least 4 weeks and stained with the Hoechst dye while alive, surprisingly, these spheres were more enriched in H33- cells (>90%, n=1) than spheres maintained in SM or SCM (Fig. 9C).

To test whether cells of the MFM spheres can maintain stemness and have multipotent properties, frozen sections made from spheres maintained in MFM for 3-4 weeks were immunolabeled for stem cell markers nestin and CD133. CD133-positive and nestin-positive cells were present throughout the sphere including the deeper region containing H33- cells and the outer layers that included H33+ cells. (Fig. 9D). We also dissociated and cultured MFM
spheres in MFM with only B-27 added (MFM-B27), SCM or SM. Immunostaining them with various stem cell and differentiation markers revealed that the cells not only differentiated but also gave rise to secondary spheres. The H33- cells identified in the SCM cultures were immunopositive for olig2, nestin, and CD133. We also identified differentiated cells in FBS which were GFAP-positive and neuron-like cells in MFM-B-27 that were positive for class III beta-tubulin (TUJ) (Fig. 12).

To analyze the circadian properties of glioma tumorspheres, C6 cells were stably transfected with mPer2::mPer2:Luc. Spheres imaged in HMFM were previously maintained in MFM for either 12 days or 4 weeks. Spheres imaged in HSCM were grown and maintained in SCM and were transferred to HSCM 5-7 days before imaging began. Spheres imaged in HSM were previously grown and maintained in SM. Using a method that synchronizes individual circadian clock cells in cell cultures [31], we applied 20 µM forskolin dissolved in ethanol (0.01% v/v) to some spheres for 2 hrs before adding 0.2 mM luciferin. SM spheres were imaged with or without prior forskolin treatment. SCM and MFM spheres were first imaged for 3-4 days without the forskolin pulse by providing only luciferin. Each sphere showed a uniform bioluminescence across its surface. Following 72-96 hours of imaging, the same cultures were treated with 20 µM forskolin for 2 hrs and then imaged in their respective media (HSCM or HMFM) for 48-96 hours. Although the same dishes were imaged before and after forskolin treatment, the same spheres were not always imaged under both conditions because they were unattached and so were displaced by the procedure. By seven days after forskolin treatment, HMFM spheres had attached to the dish and showed differentiating cells at the margins (Fig. 12).
Spheres in all media exhibited circadian rhythms in bioluminescence (Fig. 10A-C and 13), and at least 60 to 70 percent of spheres within the 20-mm diameter field of view were rhythmic with a period between 19 and 29 hrs (Table 1). Rhythms were present in all three media when spheres were treated with forskolin, but spheres in HSCM and HMFM were rhythmic without forskolin. There was no significant difference in the average period estimates for SCM and MFM spheres within the forskolin-treated or untreated groups, but the average period was significantly shorter in forskolin-untreated SCM spheres than in SM and SCM spheres of the forskolin-treated groups (Table 1). Period stability was determined from the average difference between the period estimates for the first and second circadian cycles (Table 1). By this measure, spheres imaged after 12 days in MFM had the most stable periods.

Phase analysis showed that forskolin was effective at synchronizing circadian oscillators of spheres (Table 1, Fig. 10). According to Moore’s paired test for circular data, the average phase of forskolin-treated SCM and SM spheres was not significantly different (R’=0.975, p>0.05) indicating that circadian oscillators in spheres grown in either medium can be reset similarly by forskolin. The phases of untreated and treated 12-day MFM spheres were significantly different (R’=1.212, p<0.005) indicating that the circadian oscillators in HMFM spheres can be phase-shifted and so could be capable of entraining to an appropriate daily stimulus that elevates cAMP levels. Forskolin delayed the phase by about 5.68 hrs when applied at a time near the peak of rhythms in SCM spheres (Fig. 10E) and produced a small phase advance (0.24 hr) when applied near the trough of 12-day MFM spheres (Fig. 10F).
<table>
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<th>Period (hrs)</th>
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Table I. Period and phase estimates of circadian rhythms in mper2 expression imaged in tumorspheres.
Spheres were grown in SM or SCM and then imaged in the corresponding medium (HSM or HSCM). Some spheres generated in SCM were maintained in MFM for 12 days or 4 weeks before imaging in HMFM. The average period of the untreated spheres imaged in HSCM was significantly less than the period of forskolin-treated spheres in HSCM and HSM, p<0.001(1) and p<0.05(2), respectively. Also, untreated 12-day MFM spheres were significantly shorter than treated SCM spheres, p<0.005(3) (ANOVA and Scheffe post-hoc tests). Period stability is shown by the average change in period between 2nd and 3rd cycles. Periods and changes in period are followed by S.E.M. Average phases (mean phase vectors) were determined from the time between when imaging began (or resumed after a forskolin pulse) and the peak of the first circadian cycle (converted to modulo 24 hrs) and are followed by circular S.D. All groups, except the three 4-week MFM spheres, had significant preferred phases (p<0.05) as shown by Rayleigh’s test for uniformity.
Figure 9: C6 tumorspheres enriched in Hoechst-negative cells. Live tumorspheres were stained with Hoechst 33342, fixed, and then immunostained for mitotic marker phosphorylated-serine10 of histone 3 (H3-S10p), and stained with PI. Confocal images were collected at 2-µm intervals throughout the spheres. Tumorspheres were generated and maintained in (A) 10% FBS (SM) or (B) stem cell medium (SCM). Other tumorspheres (C) were generated in 10% FBS medium and then maintained in mitogen-free medium (MFM) for 22 days before imaging. D: 40
μm-thick frozen sections of H33-stained spheres showing CD133 and nestin positive-cell population and a fluid-filled core. Bar=20 μm Bar=75 μm (A); 50 μm (B); and 100 μm (C).
Figure 10: Circadian rhythms in mPER2 expression in C6 tumorspheres. **Left:** Bioluminescence from individual tumorspheres expressing the *mPer2: luc* reporter gene grown in serum medium (SM) (A), stem cell medium (SCM) or SCM followed by mitogen free medium (MFM) for 2 weeks (C). **Center:** Bright field images of each of the three types of tumorspheres. Bar = 100 µm. **Right:** Bioluminescence from SM (A), SCM (B), and MFM (C) tumorspheres after forskolin treatment to synchronize circadian oscillators. **D:** Phases of SM spheres treated with forskolin. Phase zero corresponds with the start of imaging and the end of the forskolin treatment. **E:** Phases of all rhythmic SCM spheres without treatment (left) and after treatment (right). **F:** Phases of all 12-day MFM spheres without (left) and with (right) forskolin treatment. All phases shown are the time of the first peak of the circadian rhythm (modulo 24 hrs). (ADU-Analog digitizer units).
Figure 11: Musashi and CD133 expression in Hoechst-negative cells.  

**A:** Cells with CD133 staining (red).  

**B:** Cells with Musashi1 staining (green).  

**C:** Cells with Hoechst-positive and negative nuclear staining.  

**D:** Overlay of CD133; Musashi1 and Hoechst signals.  

Bar = 50 µm.
Figure 12: MFM spheres consist of multi-potent stem cells. 12 day old MFM spheres were incubated in SM medium, or treated with 20µM forskolin, or dissociated and cultured in SCM or differentiated in neurons with B-27 alone (MFM-B27). MFM spheres grown in SM tested positive for GFAP. MFM sphere cells cultured in SCM showed secondary spheres formation. The partially differentiated monolayer cells in SCM culture tested positive for CD133, Msi1, and olig2. Class-III beta tubulin (TUJ) positive neurons were seen in MFM-B27 culture.
Figure 13: Average circadian rhythms of Tumorspheres.  

A: Circadian rhythms from individual spheres in one HSCM dish not treated with forskolin (thick black line=average signal). B: Rhythms from other SCM spheres of the same dish after forskolin treatment. C: Average rhythms in A and B arranged with an intervening 2-hr gap during which forskolin was applied. D: Circadian rhythms from individual spheres in one HMFM dish not treated with forskolin (thick black line=average signal). E: Rhythms from other MFM spheres of the same dish after forskolin treatment. F: Average rhythms in D and E arranged with an intervening 2-hr gap during which forskolin was applied.
Discussion

Differences between CSCs and non-CSCs in cell monolayers.

The circadian properties of CSCs are unknown, and CSCs have not been identified or characterized previously in monolayers of glioma cell cultures. However, SP cells that are enriched in CSCs have been isolated from C6 cultures by flow cytometry, during which CSCs in cell suspension are selected by their exclusion of Hoechst 33342 dye. We modified the side population method by decreasing the dye concentration to identify individual CSCs in C6 monolayer cultures, H33- cells with multiple CSC attributes were observed. These cells were more rounded and significantly smaller than the H33+ cells. They proliferated in a cell culture medium that promotes stem cells growth and expressed CSC markers musashi-1, nestin, and CD133. CD133 and nestin were also detected in some H33+ cells that are probably the progenitor cells known to retain stem-like properties [94, 95]. A lack of nestin and CD133 staining in H33- cells would have challenged our hypothesis that these are CSCs, although cells negative for CD133 have CSC properties in C6 [88] and in SHG44 human glioma cells [96]. The abundance of H33- cells was estimated at 5.09% in SCM, similar to the 4% CSCs in C6 reported for cells isolated by flow cytometry using anti-nestin antibody after growth in a SCM-like medium containing B27 [89]. Animal tumor host studies with H33- cells could be used to test whether they are tumorigenic, but results would be inconclusive because both CSC and non-CSC C6 cells generate tumors [88].

Further evidence of stemness in H33- cells was provided by the distribution of a fusion protein of mPER2 and GFP, which shows cytoplasmic-nuclear movement of clock proteins [47]. The non-CSCs display a circadian nuclear localization rhythm expected of C6 cells following a forskolin treatment for synchronizing circadian oscillator cells, whereas the H33- subpopulation
is not rhythmic. Similarly, embryonic stem cells lack circadian rhythmicity before they are induced to differentiate [47, 78]. The H33- cells responded to the forskolin pulse with a significant increase in mPER2-GFP expression, indicating that their lack of rhythmicity is not due to a circadian oscillator that is refractory to synchronization. Continuous single-cell imaging [72] of H33- cells would be useful for verifying their lack of rhythmicity in these cultures.

One disadvantage of using forskolin or other agents such as dexamethasone or “serum shock” to synchronize circadian oscillators [31] is that they induce differentiation of stem cells [97] and cancer cells [98] including C6 [86, 99, 100], possibly altering cell composition during long-term imaging. We found evidence of differentiation in tumorspheres given forskolin. Because CSCs are typically only a small subpopulation within cell lines used in circadian studies, their differentiation in response to synchronizing treatments may have little noticeable influence, although studies of CSC behavior should take this into consideration. Furthermore, forskolin or cAMP elevation has other confounding effects as shown by meduloblastoma growth inhibition [101] and neuronal circadian oscillator perturbations [102]. The evidence of $mPer2$ expression in arrhythmic CSCs suggests that the protein serves a non-circadian function in CSCs or stem cells before the clock becomes active during differentiation. Similarly, the core circadian oscillator gene clock seems to function in the pancreas during early development [103]. Embryonic stem cells in culture appear to have a programmed event during differentiation when circadian rhythms appear [47]. If the clock has a causal role in differentiation rather than a mere dependency on the differentiation stage, manipulating the circadian timing system might direct CSCs to differentiate.
Imaging live CSCs in monolayer cultures could significantly enhance studies of their subcellular structures [104]. Although the Hoechst dye can cause toxicity in Hoechst-positive cells [105], any such effects on C6 H33- cells were probably minor because they excluded the dye, and it did not prevent circadian rhythms in H33+ cells. Optical access is obviously better in monolayers than tumorspheres for immunostaining or introducing probes of cell functions. Although they are a 3-dimensional configuration of cancer cells, tumorspheres in culture lack cellular stromal components found in tumors [106] or multicellular tumor spheroids implanted in mice as allograft models [107]. Individual CSCs in monolayer cell cultures receive a more uniform exposure to medium and experimental treatments than CSCs of tumorspheres. Nevertheless, additional reporter gene technologies are being developed and evaluated to more effectively probe cell behavior in tumorspheres [108].

When CSCs are identified in cell suspensions by the side population method, they are often first treated with trypsin that alters cell surface proteins. Unlike flow cytometry, the attached-cell approach minimizes cell manipulations that cause undesired effects including stress and altered gene expression and could be useful when relying on continuous live-cell imaging of CSC behavior in other cancer cell types. A method relying on aldehyde dehydrogenase activity was recently described to identify live CSCs in monolayer cultures of human esophageal cancer cells [109].

Tumorsphere circadian rhythms in three different culture media.

Although H33- CSCs in monolayer cultures showed no evidence of circadian rhythms, the tumorspheres displayed circadian \textit{mPer2} gene expression as revealed by bioluminescence imaging. The high percentage of CSCs in C6 tumorspheres, particularly ones maintained in MFM for over 4 weeks, suggested that these cells were the rhythm’s source.
Several methods for generating and maintaining CSCs from gliomas have been tested. Clonal CSC cultures from SHG44 human glioma cells have been made using stem cell and serum-based media [96]. Tumorspheres derived from glioblastoma cell lines grown in serum-containing medium were described as more enriched in CSCs than ones grown in a serum-free medium supplemented with growth factors [110]. Another study, however, suggested that CSCs derived from primary glioblastoma grown in serum-free medium containing FGF2 and EGF are more characteristic of tumor cells than glioma cell lines maintained in serum-containing medium [76]. Others reported that C6 tumorspheres grown in medium with only FGF-2 and EGF contained more side-population (H33-) cells than spheres derived from cells in serum medium, and there was no difference in CSC content between spheres grown in stem cell medium in normal versus low-attachment dishes [111]. It was previously reported that C6 cells in monolayer cell cultures maintained in a serum-free medium like MFM differentiate into cells with oligodendrocyte properties, indicating that C6 cells may behave differently in tumorspheres than in monolayers [112]. Spheres previously described as “neurospheres” made from GS-5 human glioblastoma cells by using B27 along with EGF and FGF2 in serum-free medium were reported to be enriched in neuroprogenitor cells, but the CSC content of the neurospheres was not examined. Interestingly, the neurospheres generated circadian rhythms in expression of the core clock gene BMAL1 when imaged in a serum-based medium after synchronization with a pulse of dexamethasone.

The periods of circadian rhythms recorded from spheres in the three culture media were not significantly different within the forskolin-untreated groups, but the forskolin-treated and untreated groups were different, suggesting that forskolin also lengthens period. Period stability was greatest in 12-day MFM spheres in HMFM, followed by the 4-week MFM spheres. This
result suggests that the growth factor levels in SCM perturb the circadian oscillator mechanism, perhaps through persistent activation of mitogen-activated protein kinases [113]. In contrast, the levels in MFM would be determined by secretion rates from C6 tumorsphere cells. Because CSCs can survive in spheres maintained in MFM for at least 4 weeks, they appear to compensate for the absence of supplied growth factors. The growth factors of SCM are clearly not required for circadian rhythms. Because circadian rhythms were detected in MFM spheres that were highly enriched in CSCs, as shown by H33- cells and CD133 and nestin-positive cells, it is likely that CSCs in tumorspheres can generate circadian rhythms despite their lack of rhythms in monolayer cultures. Alternatively, CSCs in spheres may be of a different type than those of monolayers. Because of their similarity to tumorspheres, gliomas within patients may also contain CSC circadian clocks, a hypothesis that these results support but that requires further testing.

C6 spheres grown in SM expressed circadian rhythms when given a synchronizing forskolin treatment, but SCM and MFM spheres expressed rhythms whether given forskolin or not. Most likely, the medium exchange synchronized clock cells in these spheres, as described previously in cell cultures [72], or the oscillators were coupled through cell interactions such as the gap junctions present in C6 [114]. Some of the rhythmic population could have included H33+ cells and these may have orchestrated rhythms throughout the sphere. Alternatively, both H33+ and H33- cells generated circadian timing signals. The state of cancer cells in tumorspheres is likely influenced by a complex microenvironment of autocrine factors and other effects from close proximity of neighboring cells and communication through gap junctions [115, 116]. A weakening of these signals in monolayer cultures may have rendered their CSCs arrhythmic.
Tumorspheres derived from glioma cell cultures have a gradient of increasing differentiation from the sphere’s core outward to the surface [117], and this pattern was observed in the C6 tumorspheres grown in SM. This distribution of H33- cells agrees with reports of a gradient of increasing hypoxia towards the core, and that hypoxia promotes the stem-like state or induces de-differentiation [118]. Further, this pattern suggests that C6 CSCs are mostly dividing symmetrically because their neighbors are usually other CSCs.

Unique properties of C6 spheres grown in mitogen-free medium.

Spheres maintained in MFM for four weeks or more after generation in SM were distinctly different from those generated and maintained only in SM or SCM. MFM spheres were nearly devoid of H33+ cells and contained a larger fluid-filled central core than in SCM spheres. They also displayed little evidence of mitotic activity as shown by sparse phospho-H3S10 immunolabeling. Spheres generated from human glioma cells in a mitogen-free medium continue to expand but at a slower rate than cells maintained in medium containing EGF and FGF2 [77]. Upon treatment with forskolin, the 12-day MFM spheres differentiated while in HMFM, indicating that their cells were viable and stem-like. In C6 monolayers, other treatments that elevate cAMP levels direct differentiation into GFAP-positive cells [119], suggesting that C6 MFM spheres also differentiated into astrocyte-like cells after the forskolin treatment.

C6 spheres may survive without added mitogens by secreting growth factors as described for other glioma tumorspheres in MFM [77]. C6 monolayer cultures are reported to produce FGF2, laminin, and fibronectin [120-122]. As in gliomas [123], autocrine factors are likely concentrated within the small interstitial spaces of spheres where they could act more effectively than in monolayers. Cells surviving within C6 tumorspheres maintained in MFM could be of a different phenotype from C6 cells in SM or SCM. For example, C6 cells in monolayer cultures
grown in a serum-free medium like MFM became rounded, were diminished in size, and
developed long branching processes [50]. Also, the doubling time increased to about 4 days,
perhaps paralleling the diminished mitosis of the C6 tumorspheres in MFM.

In summary, we conclude that H33- cells are the CSC subpopulation of C6 and lack
circadian rhythmicity in monolayers where they can be observed directly. Knowing that
tumorspheres are rhythmic qualifies them as in vitro tumor-like preparations suitable for
exploring relationships between circadian timing and cancer. The stability of tumorsphere
circadian rhythms was altered by the type of culture medium used, raising the question of
whether CSC circadian rhythms are particularly sensitive to perturbation by the growth factors of
stem cell medium. Nearly all cells in tumorspheres maintained in MFM are CSCs and they
generate circadian rhythms apparently because of sphere microenvironment properties. Similar
factors may allow CSCs within tumors to generate rhythms. Any confirmed rhythms in tumors
could be exploited with a chronopharmacological approach in which high-dosage treatments
would be given at a circadian phase when these cells are most vulnerable. Ideally this phase
could be chosen to coincide with a phase of the body’s circadian system during which damage to
non-cancer cells would be minimal.
CHAPTER III: FUNCTIONAL ANALYSIS OF mPER2 OSCILLATIONS IN MCF-7 CANCER STEM CELLS AND NON-CANCER STEM CELLS

Introduction

Disruption of circadian rhythms due to impaired expression of core clock proteins has been implicated in various forms of human cancers. One widely studied cancer type in relation to circadian rhythms is breast cancer because of the clock’s possible involvement in breast tumorogenesis. There is a growing body of evidence that links circadian disruption to the elevated frequency of breast cancer in female night shift workers. In addition to epidemiological studies showing a clear correlation between disruption of circadian rhythms and breast cancer risk, possibly mediated by the hormone melatonin, a number of studies reported that PER1 and PER2 expression in primary breast tumors is significantly decreased compared to normal breast tissues [124-126]. Expression of Per genes has also been correlated with expression of other genes implicated in breast cancers including Her2, BRACA1 and estrogen receptor [127, 128]. The deregulation of Per is most frequently associated with methylation of regions within Per gene promoters [126]. However, a large screen of human breast cancer genomes identified mutations in both Per1 and Per2 genes, supporting their role as tumor suppressors [129]. These studies were conducted in both primary breast tumors isolated from patients as well as breast cancer cell cultures. 

Of the several available breast cancer cell lines is the widely studied MCF-7 line, which was isolated from an adenocarcinoma at a metastatic site of a 69-year-old female. Two studies have reported lack of Per2 expression at both the transcript and protein levels in MCF-7 cells cultured in SM [128, 130]. Another study reported that overexpression of a Per2 transgene leads to tumor suppression in mice and increased apoptosis via p53 activation in cultured MCF-7 cells
How Per2 overexpression leads to p53 activation is not clear. A 2013 report indicated that p53 negatively regulates Per2 expression [132], thus describing a possible homeostatic mechanism through which PER2 protein levels are kept within a particular range by a negative feedback loop and further linking this clock gene to the tumor suppressor p53.

Although several cancer cell lines have distinct circadian rhythms in gene expression, the reason why many other cancer lines apparently lack circadian clocks remains unclear. Similarly, circadian rhythms of cells within tumors are often poorly organized or absent (David Weaver on cancer and circadian rhythms). Considering the cell heterogeneity of cancer cell lines, including cancer stem cells within these lines, it seemed likely that some of the cells could retain a functional circadian clock that was not obvious when gene expression in entire cultures was examined. All the studies that have investigated circadian rhythms in MCF-7 cells have cultured them in serum containing medium. There is much evidence suggesting that cells cultured in serum medium consist of a more differentiated population and a small CSC population. Thus, it can be argued that previous studies that investigated circadian rhythms in MCF-7 cells using real time PCR mostly recorded properties of differentiated cells and failed to adequately describe gene expression patterns in the CSC population. Based on this information and our previous work on C6 glioma stem cells, we hypothesized that Per2 expression may be differentially regulated in stem and non-stem-like MCF-7 cells.

To address this concern, we probed the circadian properties of MCF-7 cultures with a reporter gene that expresses a functional mPER2 protein fused with firefly luciferase under the control of the mouse Per2 promoter, the construct we used in C6 cells. MCF cells grew as small clusters in medium containing FBS or one containing growth factors (FGF2, EGF, and PDGF) to stimulate CSC proliferation. As described in Chapter 2, we found that the C6 glioma cell line
produces robust circadian rhythms when grown as tumorspheres. Cancer cell subtypes, such as CSCs, can interact within the tumor microenvironment and may produce circadian timing signals independent of any disorganized timing in the rest of the tumor. Additionally, by using end point RT-PCR we also analyzed the expression levels of hPer2 in unmodified MCF-7 cells cultured as spheres in SCM. Our findings suggest that circadian mPER2 oscillations are present in MCF-7 stem cells, have higher amplitude than in spheres maintained in SM, and that hPer2 expression may be differentially regulated in two subpopulations of MCF-7 cells.

**Materials and Methods**

Cell culture, transfection, and reporter gene assay.

MCF-7 cells were a kind gift from Dr. Kathryn Eisenmann at the University of Toledo School of Medicine and cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% FBS and with no pyruvate or phenol red (serum medium, SM) at 37°C in 5% CO2. Cells were either transfected with mPer2::mPer2:luc or cotransfected with mPer2::mPer2:luc and CMV::neo to produce a fusion protein containing mPER2 and firefly luciferase [34] by following a standard transfection protocol (Fugene HD transfection reagent, Promega). In the first case, transfected cells remained unselected and continued to grow in SM or SCM medium. Cotransfected cells, on the other hand, were selected with G418 (1 mg/ml) in DMEM and 10% FBS (v/v) for 7-10 days. Selected clones were tested for expression of luciferase. Positive clones were isolated in 35-mm dishes and grown in either SM or a stem cell medium (SCM) containing 20 ng/ml EGF; 10 ng/ml bFGF2; and 0.5X B-27 in DMEM. In both cases cells were imaged in HEPES-buffered SM and HEPES-buffered SCM with reduced bicarbonate levels, as in Chapter 2, with 200 μM luciferin, Penicillin and streptomycin were added to all media.
Fluorescence and bioluminescence imaging.

Fluorescence microscopy and bioluminescence imaging of MCF-7 cultures was performed as described in Chapter 2 in which C6 tumorspheres were imaged. Fluorescence imaging was performed with either a multiphoton laser-scanning confocal microscope at the University of Toledo, a spinning-disk confocal microscope (CREST) at BGSU or a wide-field fluorescence microscope (Zeiss Axiophot) at BGSU.

Promoter sequence identification and alignment.

Promoter sequences for both hPer2, Gene ID-8864, reference sequence NC_000002.12 (239211190-239214190), mPer2, Gene ID- 18627 and reference sequence NC_000067.6 (91661521-91664521), complement were identified using TRED database [133, 134]. The 2500 bp upstream regulatory region and 500 bp downstream coding sequence (-2500 to +500) of both promoters were used for alignment. Alignment was performed using Clustal W2 aligning software [135].
Results

BLAST analysis of mPer2 and hPer2 gene promoters.

Previous reports on circadian rhythm analysis in MCF-7 suggested that these cells do not exhibit a functional oscillator due to lack of Per2 expression at the protein and mRNA levels [128, 130]. It is important to note that the studies in both cases were performed with MCF-7 cells cultured in medium containing serum (FBS or fetal calf serum). Additionally, the cells in culture were synchronized with a serum shock method which uses a pulse of high serum concentration in medium. MCF7 cells are well characterized for their heterogenic population and when grown in presence of FBS contain mostly differentiated cells and only about 1-4% CSC as determined from the CD44$^{high}$/CD24$^{low}$ ratio. Any Per2 gene expression rhythms may have been below detection by transcript level analysis. In contrast, MCF-7 contains 40% to 50% more CSCs when grown in SCM [136]. Our previous report on C6 tumorspheres suggested that stability of tumorsphere circadian rhythms is altered by the type of culture medium used, possibly because of a sensitivity of the clock to the growth factors in the medium. We also observed in the same study that nearly all cells in tumorspheres maintained in MFM are CSCs and that they generate circadian rhythms apparently because of sphere microenvironment properties. Consequently, we tested whether MCF-7 cells exhibit circadian rhythms in SCM while growing as clusters or mamospheres (breast cancer cell tumorspheres).

Because MCF-7 cells are of human origin and our reporter construct contained the mouse Per2 promoter, we performed a sequence alignment analysis between hPer2 and mPer2 to compare the sequence identity of the two promoters. There was sequence alignment of 62.27% and 66.28 % sequence identity (Fig. 14). The scores suggest that the Per2 promoter is highly
conserved between the two species. The amino acid sequence of \textit{mPer2} exhibits 78\% identity overall to that of \textit{hPer2} [137]. The two sequences not only had high sequence identity but were also 100\% identical in the E-box flanking sequences. To upstream flanking sequence seems to be a potential CpG island which may be subject to promoter hypermethylation leading to the silencing of the Per2 promoter expression. The identical flanking sequence and the E’-box sequences have been reported by two other research groups [27, 132]. Based on these results we concluded that using the \textit{mPer2::mPer2:luc} reporter construct in MCF7 cells should provide expression similar to that of the endogenous \textit{hPer2} promoter.
Mouse Per2, chr1; TSS: 91664021 [-2500..+500](+strand)
tgctctctctggccccctccaaactctgtgagtagtagggcaccacaacactgtctgtgacagtctagtttctttgcatagcatggcagcaaatagttttagttcggagagagtctgctgtcttcagggctgaagagatagtttagctgtttaaaaaacattattcccttacaggtctctttgcaagctcacaacaatctataactcaagccagtgtgaggatacagtgtaaaaatccaaaccgggtgggcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctgttttaaaaaacactgtgcttcagggctgaagagatagtttagctg
Human PER2, chr2; TSS: 239213690 [-2500..+500](-strand)

agctgtaggaggtggctggaggtggtgcaagcaggtgtttctgtgtcttgcggtgccttagagttgggag
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Figure 14: *hPer2* and *mPer2* promoter sequence alignment. Shaded regions show the predicted E’-box (red) in the *hPer2* and *mPer2* promoters which lies ~20bp upstream of the transcription start site. Aligning Score = 62.27; Sequence identity Score = 66.28; shaded region green = Downstream (+500 bp); blue = Upstream (-2500 bp); yellow = 100% alignment of CACGTT flanking sequences; pink = 100% alignment of CACGTT flanking sequence and...
potential CpG island. This sequence is identified by TRANSFAC as USF binding element for both human and mouse promoter; Purple = Canonical E-box in Human promoter that positional weight matrix algorithm of TRANSFAC identifies with high score. The CACGTT E’-box has been reported earlier by two independent groups [27, 132]
The mouse Per2 (mPer2) promoter is induced in MCF-7 cells. Because the reporter construct does not contain a eukaryotic selectable marker the cells were left in the transfection medium for 24 hrs and were replaced with fresh medium and allowed to grow for another 48 to 72 hrs before being transferred to fresh HEPES-buffered DMEM containing 200 µM luciferin. The cells grew as a monolayer and small clusters in SM, but as spheres in SCM medium.

To test whether the clusters growing in SCM medium consisted of CSCs, we immunostained untransfected MCF-7 cultures growing in SM and SCM medium with reported breast cancer stem cell markers CD-133 and Msi-1 [138, 139]. We observed that clusters growing in SCM medium expressed both Msi-1 and CD133 in comparison to the MCF-7 cells growing in SM medium that did not express CD133 but weakly expressed the Msi-1 marker, suggesting that the MCF7 sphere cultured in SCM contains stem like cells (Fig. 15). Both clusters and spheres of MCF-7 cells in either SM or SCM expressed Per2-luciferase fusion protein as early as 24 hrs post transfection, suggesting that all transcription factors and clock proteins that are required to activate the mPer2 promoter are present in MCF-7 cells (Fig. 16). This result also suggests that MCF-7 may contain a functional clock but with an amplitude too weak to be analyzed at the transcript level using qPCR in previous studies. Alternatively, it is possible that the endogenous Per2 promoter in MCF7 cells cultured in the presence of serum is transcriptionally silent due to promoter hypemethylation or post transcriptional mechanisms.

When we tested for circadian oscillations the MCF-7 clusters and spheres in SM and SCM medium, respectively, exhibited distinct circadian oscillations. Clusters in SM exhibited an average period of 23.05 hrs (SEM ±0.96 hrs, n=48, from 2 cultures), and clusters and spheres in SCM exhibited an average period of 22.58 hrs (SEM ±0.51 hrs, n=77 spheres and clusters from
3 cultures, Fig. 17). Of the total clusters and spheres analyzed, 56.25% of clusters in SM were rhythmic and 74% of clusters and spheres in SCM were rhythmic. Interestingly, we observed a significant difference in amplitude of rhythms measured in SM and SCM cultures (one-way ANOVA for SM, \( n = 24, \text{mean} = 72.70, \text{SEM} = 10.06 \text{ ADU} \); SCM, \( n = 19; \text{mean} = 913.15, \text{SEM} = 119.93 \); and SM to SCM, \( n = 30, \text{mean} = 200.83, \text{SEM} = 16.49 \)) (Fig. 18).
Figure 15: MCF-7 cells express stem cell markers in SCM medium. **Left**: fluorescence. **Right**: overlay of fluorescence with brightfield images.
Figure 16: MCF-7 cells expressing the \textit{mPer2::mPer2:luc} reporter construct.

Bioluminescence image of Numbered locations indicate objects where rhythmicity was analyzed by a region-of-interest placed over the spot to quantify expression. MCF-7 cells cultured, transfected, and propagating in SM (A) or in SCM (B). Note the higher overall signal in B and more distinct objects.
Figure 17: *mPer2* exhibits circadian oscillations in MCF-7 cells. Cells cultured in SM (A), SCM (B), or cultured in SM, imaged in SM, and then transferred to SCM and imaged in SCM 72 hrs later (C).
Figure 18: Circadian amplitude estimation of clusters and spheres expressing the *mPer2::mPer2:luc reporter gene*. Estimated amplitudes of rhythms in SM and SCM are significantly different (*). Amplitudes estimated for a culture growing in SM (96 hrs) followed by transfer to SCM (120 hrs) showed a significant increase in amplitude. (p <0.001* and F = 61.73 between SM and SCM and F = 8.50 between SM and SM to SCM cultures).
hPer2 gene is differentially expressed in MCF-7 cells.

Based on previous reports MCF-7 cells grown in serum medium did not exhibit circadian rhythms in Per2 mRNA levels. However, we observed rhythms in mPer2 expression in MCF-7 cells growing in SM and SCM with more robust rhythms in the MCF-7 spheres formed in stem cell medium. Due to more robust expression of mPer2 in SCM medium and based on our observation that C6 SCM spheres exhibit stable rhythms compared to the SM spheres we asked if there was a differential expression of the Per2 gene in MCF-7 cells cultured in SCM compared to ones cultured in SM. We extracted total RNA from non-transfected MCF-7 cells cultured as monolayer in SM and as spheres in SCM. Using primers specific for hPer2 we performed an end point RT-PCR to detect the expression levels in two different populations. We observed that hPer2 was expressed at higher levels in the spheres (Fig. 19). We also checked the expression of cancer stem cell self-renewal gene Bmi-1. Bmi-1 expression was also found to be elevated in MCF-7 spheres but was expressed in both populations. Based on this data we concluded that Per2 may be expressed at different levels between monolayer cells representing the more differentiated population and spheres representing the more stem-like population.
Figure 19: Expression of hPer2 in MCF7 monolayer cells and sphere cells. 100 ng of total RNA was used for the analysis of hPer2 and Bmi-1 expression in MCF-7 monolayers and spheres.
Discussion

MCF-7 cells growing in SCM formed clusters that produced circadian rhythms. Cluster formation in SCM is characteristic of CSCs. Therefore, we conclude that, like C6 CSCs, MCF-7 CSCs may require close cell interaction, as within tumors, to generate rhythms. Monolayer cultures were examined in previous studies of clock gene expression in MCF-7 [128, 130], in which case the CSCs may be arrhythmic, as in C6. We found areas outside the cell clusters that produced bioluminescence and therefore were able to activate the \( m\text{Per2} \) transgene, but we cannot conclude that the monolayer cells contain circadian clocks. Unlike C6 monolayer cells, circadian rhythms in the non-CSCs may be absent in MCF-7 cells. Alternatively, The MCF-7 cells may have weak rhythms that may not be detectable using qPCR methods. Although additional circadian studies are needed, this distinction could be a fundamental difference between other breast cancer cells and glioma cells, perhaps because of the very different tissues from which they originate. An examination of a second cancer cell type of ectodermal origin, such as the melanoma cell line D10 which is known to consist of a significant CSC population [140], should be compared with the circadian properties of C6 CSCs and non-CSCs described in these experiments.

One similarity between MCF-7 and C6 that was clear is the significantly elevated \( m\text{Per2} \) expression in cells maintained in SCM rather than SM during imaging. It is likely that the growth factors used in SCM induced the \( m\text{Per2} \) promoter. Previous studies showed activation of \( m\text{Per2} \) through MAPK pathways. We concluded in Chapter 2 that stimulation though this pathway acts as an input to the clock mechanism in C6, but may be excessive for the tumorsphere circadian clock. It reduced rhythm stability as evidenced by the smoother and more stable rhythms of C6 tumorspheres in MFM that lacked any added growth factors. Similarly,
MCF-7 mammospheres should be imaged in MFM to determine whether circadian rhythms are improved relative to SCM cultures. Because many mammary tumors are responsive to estrogens, effects of these hormones and their receptor agonists should also be tested for effects on circadian rhythm amplitude or stability in preclinical studies of possible therapeutic strategies.

Perhaps, the most important outcome of these experiments is the recognition that MCF-7 cells can express circadian rhythms. The vast amount of data collected on MCF-7, including the role of CSCs in EMT and metastasis [141], can now be evaluated in relation to circadian regulation of cancer cell proliferation, metabolism, cell death, drug sensitivity, etc. Rhythmic cells have many clock-controlled genes, perhaps as much as 20% of the genome that act through multiple cell signaling pathways to regulate much of cell physiology. In contrast, an arrhythmic cancer cell would not benefit from the ability to predict rhythmic events in the body, such as the onset of increased immune surveillance at night or heightened insulin production following meals and its growth-promoting effects. Whether some cancer cells experience selective pressure to retain a circadian clock, despite major chromosomal disruption and metabolic peculiarities, remains an interesting unresolved question.

We observed a significant difference in the amplitude of the oscillations of $mPer2$ gene expression between the monolayer cells and spheres. Interestingly we also observed elevated expression of $hPer2$ expression between the two cell types, and an interesting hypothesis can be generated from this observation: One possible explanation for the amplitude difference is that $Per2$ transcripts in monolayer cells, which are mostly differentiated cells, is post-transcriptionally regulated. Post-transcriptional regulation can occur in various ways including inhibition of translation by RNA-binding proteins, thereby reducing mRNA degradation via miRNA. A first indication of a circadian clock gene being regulated at the post-transcriptional
level came from studies of Per gene transcript stability [142]. However, only in 2009 was it first shown that miR192 regulates the period gene family (Per1, -2, and -3) mRNA stability, in turn affecting the phase and amplitude of the oscillation[143]. Interestingly, miR192 is a member of the miR200 family of micro RNAs that are known to participate in control of cell cycle and cell differentiation. Also, miR192 is negatively regulated by transcription factor ZEB/SIP1 during EMT. ZEB1/SIP1 is required for the mesenchymal state and is absent in epithelial cells, thus allowing miR192 expression [144]. MCF-7 cells in monolayers are analogous to epithelial stem cells, whereas CSCs of the sphere are more like mesenchymal stem cells.

Furthermore, miR192 has been implicated in inducing stem cells to differentiate into endodermal progenitor cells [145]. One study showed that expression of miR192 in E19 mice is involved in scarless tissue formation by allowing differentiation of mesangial cells, indicating interaction with stem cells. Also, miR192 has also been reported to induce mesenchymal stem cells to form astrocytes [146]. Additionally, miR192 is involved in targeting proteins regulating cell proliferation, IGF activity and p53 activity [147]. In addition to miR192 another micro RNA, miR141, has been shown to influence core circadian gene CLOCK at the post-transcriptional level [148, 149]. A decrease in CLOCK protein levels may lead to decreased activity of BMAL1/CLOCK and inhibit transcription. One study reported that targeting miR192 by using antisense RNA reduced both miR192 and miR141 [150]. miR141 is also involved in targeting transcription factors required for maintenance of stemness and pluripotency [151]. Based on these facts and our observation of differential hPer2 expression in the MCF-7 monolayer and spheres a model can be proposed describing the role of miR192 and miR141 in regulating the amplitude of Per2 oscillations in differentiated cells. Similarly, low expression of miR192 and
miR141 in stem cells may allow more robust circadian oscillations, thus allowing stem cell genes to be expressed more dynamically under control by clock proteins (Fig. 20).

These results with MCF cells also establish a theoretical basis for treating breast cancer through chronopharmacology, an approach that uses circadian timing to deliver the highest tolerable chemotherapeutic agents when cancer cells are most vulnerable and, ideally, when non-transformed cells are most resilient. Also, the effects of disrupted melatonin rhythms on breast cancer risk in night-shift workers, a likely but not proven cause of malignancy, can be better understood and perhaps better mitigated by further examination of circadian clocks within breast cancer cells.

A question remains about the three previous reports describing the activity of the Per gene in MCF7 cells. In two of these studies, Per2 mRNA was not expressed at levels detectable by qPCR [126, 128]. In a third study, hPer2 was measurable and induced by application of progesterone, which also induced hPer2 in reproductive organs [152]. One possibility is that the hPer2 gene is partially or completely silenced in MCF-7 by mutation or epigenetic modifications, which is in agreement with purported tumor suppressing abilities of PER2 protein in various cancer cells. However, PER2 shows distinct expression and circadian rhythmicity in C6 and other cancer cells that are capable of robust tumor formation, indicating that loss of the circadian clock is not required for cell transformation or cancer progression. Nevertheless, the transgene we introduced into MCF-7 may have rescued the cells from an arrhythmic state, in which case all other studies of MCF-7 have been performed with cells lacking a circadian clock. An analysis of the hPer2 promoter and structural gene sequence in MCF-7 could be useful for detecting a loss of function. A concomitant loss of function of the hPer1 gene of the period family would, however, be expected to explain complete loss of clock functions, because a
knockout of both alleles of the \textit{mPer2} gene do not cause loss of circadian rhythms in mice, whereas a double knockout of \textit{mPer1} and \textit{mPer2} does.

Whether or not there was a rescue by the transgene, the trans-acting factors that regulate \textit{hPer2} are intact in MCF-7, because they did regulate the transgene rhythmically. If there was a rescue, the transgene’s promoter escaped any methylation that might have silenced \textit{hPer2} at CpG islands we and others have identified in the promoter sequence. Furthermore, it would be quite remarkable that an introduced clock gene could rescue an arrhythmic cancer cell because this approach might be used to awaken latent circadian clocks to alter cell behavior. It may be an effective way to suppress malignancy by restoring tumor suppression through PER2.
Figure 20: Model explaining miRNA 192 and 141 regulation of clock amplitude and stem cell differentiation. In stem cells the expression of miR192 and miR141 may be suppressed by mechanisms and pathway not yet identified in MCF-7. This suppression could allow Per2 and Clock transcripts to be stabilized, leading to robust expression of CSC genes. In differentiated cells the transcript levels of Per2 and Clock may decrease because of miR192 and miR141 expression by an amount that affects the amplitude of Per2 and transcription functions of Bmal1 proteins.
CHAPTER IV: ANALYSIS OF CIRCADIAN REGULATION OF STEM CELL GENES IN GLIOMA AND BREAST CANCER CELLS

Introduction

What allows CSCs to survive for so long or become resistant to chemotherapeutic drugs is still unclear. Research on signaling pathways that contribute to CSC survival has, however, identified some important pathways that may underlie survival, progression and resistance, of glioma, breast and other CSCs. Self-renewal of normal and malignant stem cells of humans or mice involves a diverse network of regulatory mechanisms, including the signaling pathways of Notch, Hedgehog, Wnt/β-catenin, epidermal growth factor (EGF), platelet derived growth factor (PDGF)[153-155], leukemia inhibitory factor (LIF) [156], and TGF-β [157]. The Notch, Wnt, Hedgehog, and HER2 signaling pathways and the polycomb-group (PCG) transcription factor BMI-1 govern the differentiation and self-renewal of normal neural and breast stem cells. Furthermore, their deregulation is known to promote cancer [158-161]. The Notch signaling pathway is implicated in cell differentiation and self-renewal of both glioma and mammary stem cells. Over-expression of the active form of Notch 4 inhibits differentiation of breast epithelial cells and might contribute to breast carcinogenesis [153, 162]. Musashi-1 (Msi-1) is a positive regulator of Notch signaling, and both Msi-1 and Notch 1 are key regulators of asymmetric cell division in human epithelial stem cells [139, 163-165]. The Hedgehog pathway is required for normal and neoplastic development of the mammary gland. The PCG transcription factor BMI-1 is overexpressed in human cancer cell lines more specifically in glioma and breast cancers. BMI-1 effects on stem-cell renewal appear to involve epigenetic silencing of the p16\(^{INK4a}\) gene. BMI-1 impacts self-renewal of normal and malignant human glioma and breast stem cells by activating Hedgehog signaling pathways [158, 166, 167]. Based on their importance and relevance to
glioma and breast CSCs we chose the components of the following pathways for the analysis of their circadian regulation.

**Notch and HES-1 signaling pathways in cancer stem cells.**

Activation of the protein HES-1 in cancer cells occurs in response to signals from outside the cell that bind to the Notch protein on the surface of cancer cells [83]. HES-1 is a member of a family of proteins that regulate the development of the brain by controlling the pattern of cell division [84]. It is also produced in normal stem cells of the adult brain that help in replacing neurons and the other brain cells (glia) [49]. HES-1 was selected for this study because it appears to be positioned between the circadian rhythms found in cancer cells and the control of cell division rate. Obviously, potential therapies that can act on a protein that regulates cell replication could be valuable for controlling tumor growth. HES-1 is known to undergo feedback that represses the *Hes-1* gene promoter, thereby creating an ultradian oscillation that times the formation of embryonic structures such as somites from the mesoderm.

Interestingly, *Hes-1* gene expression has been shown to be required for the self-renewal of neural stem cells as it represses the differentiation of these cells into neurons and thus allows the maintenance and persistence of the stem cells. More importantly, our promoter analysis of the *Hes-1* gene, using the TRED database, identified a canonical E-box (CACGTG) element, suggesting influence of clock components on *Hes-1* gene expression. Furthermore, HES-1 appears to be regulated by binding to the protein ID-2 [50, 85], a product of a circadian clock-controlled gene.

We speculate that the molecular circadian clock is functional in H33-positive cells but not in H33-negative cells of monolayers, Furthermore, we hypothesize that the clock serves in a
circadian repression of Hes-1 in tumorspheres and that this repression throughout about half of the day is adequate to prevent GSCs from differentiating. The possibility of inhibiting HES-1 through its regulation by the circadian clock provides an opportunity for forcing GSCs to differentiate and so become cells that are more vulnerable to chemo-therapy.

Hedgehog; Gli-1 and Bmi-1 signaling pathways in cancer stem cells.

The sonic hedgehog (Shh) pathway is considered a key component of nervous system development by modulating precursor cell proliferation. More importantly, recent studies have shown a strong correlation between a more active Shh pathway and GBM patient survival. The Gli-1 transcription factor is primarily responsible for activation of the Shh pathway which in turn activates Bmi-1, which then promotes stem cell self-renewal by maintaining repression of p16\textsuperscript{Ink4a} and p19\textsuperscript{Arf}. Elevated Bmi-1 expression was detected in 99% of the breast tumors tested. Also, it has been shown that Bmi-1 is highly expressed in human GBM and breast cancer samples, and that Bmi-1 deficiency reduces the invasiveness of malignant mouse astrocytes carrying a null mutation in the Ink4a/Arf locus. Additionally, our promoter analysis revealed that the Bmi-1 promoter may contain a canonical E-box element, suggesting that this important stem cell self-renewal gene may be under clock regulation. Taking all the evidence together and considering our hypotheses, it is possible that a molecular circadian oscillation in H33-negative cells may, at different phases of the cycle, allow either differentiation or quiescence and self-renewal, thereby contributing to growth and persistence of glioma tumors by providing a temporal ordering of these cancer stem cell processes.
Materials and Methods

Cell Culture and Sphere formation.

C6 cells were cultured in complete DMEM medium containing 10 ng/ml FGF-2, 20 ng/ml EGF, and 10 ng/ml PDGF. MCF-7 cells were cultured in complete DMEM medium containing 20 ng/ml of FGF-2 and 20 ng/ml of EGF. All three cell types were cultured in a standard tissue culture dish. Floating spheres (220-400 µm in diameter) were harvested about 2 weeks after cell dispersal.

Promoter sequence identification and transcription factor binding site identification.

Gene promoter sequences were identified using the Transcriptional Regulatory Element Database (TRED). The 3000 bp upstream regulatory region and 1000 bp downstream coding sequence (-3000 to +1000) of promoters were used for identification of predicted transcription factor binding sites. Transcription factor binding search was restricted to upstream stimulatory factor (USF, the canonical E-box) and aryl hydrocarbon receptor nuclear translocator (AHR, Arnt). The predicted elements in the regulatory region of the gene promoters were identified using TRED and TRANSFAC databases. Both databases use a positional weight matrix algorithm to predict the transcription factor binding site. The matrix search parameters were set to retrieve only high quality matrix results and to minimize false positives. The predicted sites were compared with the Encyclopedia of DNA Elements (ENCODE) database of transcription factors identified by ChIP sequence. The prediction was considered a positive hit only if the three databases confirmed the presence of the transcription factor site.
RNA extraction and cDNA synthesis.

Total RNA was isolated from C6 and MCF-7 tumorspheres. Total RNA was isolated using Arcturus Pico Pure RNA Isolation Kit from Life Technologies. The spheres were grown for 2-3 weeks in their respective SCM (20 ng/ml EGF; 10 ng/ml PDGF and 10 ng/ml FGF for C6 and; only EGF and FGF for MCF-7). Spheres were transferred to a single dish and given a 20 µM forskolin pulse for two hours. At the end of two hours, about 30-40 spheres were distributed into each of individual 35-mm dishes. Total RNA was collected every 3 to 6 hrs for 48 hrs. The isolated RNA concentration was measured using a nanodrop spectrophotometer (Thermo Scientific) and quality was assessed on a 2.5% agarose gel. The RNA was stored at -80°C until used for cDNA synthesis. A total of 2 µg of RNA for each time point was used for first strand synthesis using a high capacity cDNA synthesis kit from Applied Biosystems. The cDNA was synthesized at 37°C for 120 min, followed by a 5-min incubation at 85°C. The cDNA was stored at -20°C until the analysis.

Quantitative real time polymerase chain reaction (qPCR).

PCR primers used for this experiment are listed in Table 2. An initial DNA denaturation step at 95 °C for 2 min was followed by 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 53 to 58 °C for 15 s, and extension at 72 °C for 30 s. The primer specificity was confirmed by sequence searches in human and rat DNA databases (NCBI Primer search) and analyzed electrophoretically on agarose gels. qPCR was performed using a Bio-Rad MJ Mini Opticon thermocycler apparatus (Bio-Rad) and Bio-Rad CFxX Manager software (Bio-Rad). All PCR procedures were performed in duplicate in a volume of 20 µL using 48-well optical-grade PCR plates and an optical sealing tape (USA Scientific). The expression of all clock and CCG
transcripts was normalized with respect to GAPDH for MCF-7 and β-actin for C6. The transcript levels for each time point were calibrated to the 0 hr time point and calculated using the formula $2^{-\Delta\Delta C_t}$.
<table>
<thead>
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<td></td>
<td>Reverse</td>
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Table 2: List of primer pairs used in real time PCR analysis of clock regulation in cancer stem cell genes.
Results

The promoters of cancer stem cell genes may be clock regulated.

We observed robust circadian rhythms in the tumorspheres of C6 glioma and MCF-7 cancer cell lines. Based on this observation we asked if the cancer stem cell self-renewal genes were clock regulated. To identify any possibility of the cancer stem cell genes being clock regulated we performed insilico promoter analysis of gene promoters of candidate stem cell genes. Based on the relevance of their expression in both glioma and breast cancer cells we chose *Hes1* and *Bmi1* for our analysis. Using a four-point validation approach (Fig. 21, Table 3) we determined whether the promoter of the candidate stem cell self-renewal genes consist of an upstream stimulating factor (USF) or an aryl hydrocarbon receptor nuclear translocator (AHR/Arnt) binding elements. USF contains a leucine repeat that is required for efficient DNA binding. USF binds the core sequence CACGTG (E-box). Aryl hydrocarbon receptor, a transcription factor that acts in circadian behavior in humans, is associated with several cancers including breast and brain.

The four-point validation approach uses a positional weight matrix (PWM) algorithm with high matrix score and minimized false positives. The candidate transcription factor binding elements were identified by using a PWM matrix from two independent databases TRED and TRANSFAC 2014.1. The promoter sequence was then fed into the ENCODE database that identifies the transcription factors based on ChIP sequence data submitted by various sources. Followed by identification, the rhythmic expression of the candidate gene was verified from the CIRCA database, which is a collection of various cDNA microarray analyses of rhythmic expression of genes in different tissues obtained from mice, humans, or from various cell lines.
(NIH 3T3). Using this approach we determined that both candidate gene promoters contain a putative E-box element (USF or AHR) and are rhythmically expressed in at least one animal tissue (Table 3).

Circadian regulation of Hes1 and Bmi1 in C6 and MCF7 cancer stem cells.

To address the question of whether the cancer stem cell genes *Hes1* and *Bmi1* are under clock regulation we performed qPCR analysis of their transcripts at 6-hr intervals for 48 hrs. To correlate the gene expression analysis with our bioluminescence data we harvested total RNA from only 20-30 spheres at each time point. A 2-hr, 20 µM forskolin pulse that was used to synchronize the *mPer2* reporter gene rhythms in spheres used for bioluminescence imaging was also used to synchronize spheres for total RNA extraction. The spheres were harvested starting at 24 hrs after synchronization to avoid any acute effects of the forskolin pulse. The quality of RNA at each time point was checked for RNA integrity using gel electrophoresis. Only if the RNA was stable and no degradation was observed was it used for cDNA synthesis followed by qPCR analysis. As positive controls we used primers for clock genes *Per2*. The gene expression was normalized to *Gapdh* for gene expression in MCF-7 cells and *β-actin* for gene expression in C6 cells (ΔCt). After normalization the transcript levels at each time point were calibrated to 0 hr time point (ΔΔCt). Using the formula $2^{-\Delta\Delta Ct}$ the mRNA levels at each time was calculated. Based on the results obtained, we observed a near 24-hr oscillations in the transcript levels of *rPer2* and *hPer2* in C6 and MCF-7 tumorspheres, respectively (Figs. 22 & 23). However, we did not observe a 24 hr oscillation in the transcript levels of *rHes1* in C6 tumorspheres. Instead, C6 *rHes1* expression exhibited faster oscillations.
Figure 21: **Four-point validation for in silico analysis.** Flowchart describing the four point validation method for in silico identification of putative, functional E-box elements in the candidate gene promoters.
<table>
<thead>
<tr>
<th>Gene Promoter</th>
<th>TRED Promoter Sequence Retrieved</th>
<th>TRED Matrix Search for USF (E-box)</th>
<th>ENCODE Database</th>
<th>TRANSFAC 2014.1 Prediction AHR (CACG/TC/T_)</th>
<th>CIRCA Database output (Lomb-Scargle, p&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hes1</td>
<td>Human, Mouse and Rat</td>
<td>-306 (CACGTG) PWM</td>
<td>USF1 (CACGTG) 411 bp upstream pol2 binding site</td>
<td>-366 bp on plus strand PWM</td>
<td>Tissue Expressed = Distal colon Period = 6.7 hr</td>
</tr>
<tr>
<td>Bmi-1</td>
<td>Human and Mouse</td>
<td>-200 bp (CACGTG) PWM</td>
<td>USF1 (CACGTG) 563 bp upstream pol2 binding site</td>
<td>-300 bp on minus strand PWM</td>
<td>Tissue Expressed = Kidney, Period = 26.7 hr</td>
</tr>
</tbody>
</table>

Table 3: Analysis of USF and AHR transcription factor binding sites on the promoters of cancer stem cell candidate genes. Using the TRED matrix and TRANSFAC database E-boxes were identified at the indicated positions upstream of the start sites.
C6 rBmi-1 expression exhibited oscillations with periods that were greater than 24 hours. However, the first and the third peaks of rBmi-1 in C6 aligned with the first and third peaks of the rPer2 results. The second peak of rBmi-1 was relatively small, but it still aligned with the second peak of the rPer2 data. One possibility is that there was an error in RNA loading and less RNA was used for determination of the second rBmi-1 peak.

In the case of MCF-7, we observed a 24-hr oscillation in the expression of hPer2 (Fig. 23). However, we could not detect a circadian oscillation in the expression of hBmi-1 in MCF-7 tumorspheres, suggesting that hBmi-1 in MCF-7 cells may not be under direct clock control.
Figure 22: Preliminary results of a real time analysis of tumorsphere mRNA expression in C6. C6 spheres exhibit circadian $rPer2$ expression after forskolin treatment (at time 0) to synchronize clock cells. The stem cell genes $rHes1$ and $rBmi1$ do not appear to be under strong circadian control after forskolin treatment and they exhibit faster oscillations.
Figure 23: Preliminary results of a real time analysis of tumorsphere mRNA expression in MCF-7. MCF-7 spheres exhibit circadian hPer2 expression after forskolin treatment (at time 0) to synchronize clock cells. The stem cell gene hBmi1 does not appear to be under strong circadian control after forskolin treatment and it exhibits faster oscillations.
Figure 24: Comparison of mPER2 protein and transcript oscillations in C6 tumorspheres.

The mPER2 protein expression (above) exhibits 3-6 hours of delay when compared with \textit{mPer2} transcript oscillations (below) in C6 tumorspheres after forskolin synchronization.
**Discussion**

It is well established that cancer stem cell genes *Hes1* and *Bmi1* are required for self-renewal of both normal and cancer stem cells. Knock down of these genes leads to loss of self-renewal of the stem cells and is known to cause developmental defects in the brain and other parts of the embryo (168 and 169). Because we observed robust circadian rhythms in the tumorspheres formed by C6 and MCF-7 cells we asked whether *Hes1* and *Bmi1* are clock regulated. We detected rhythms in clock genes but did not detect circadian oscillations of the cancer stem cell genes. As predicted, the phase of the peaks in *mPer2::luc* bioluminescence rhythms followed closely behind the peaks observed in the *rPer2* gene expression rhythms (Fig. 24) confirming the bioluminescence results in this study.

These preliminary data, based on only single samples at each time point, suggest that neither of the cancer stem cell genes is under direct or indirect clock control and may exhibit a clock-independent mechanism of regulation in CSCs. If the results obtained here do not change substantially after sufficient replication at each time point has been performed, we could reject models that include significant timing interactions between circadian rhythms and stem cell gene activity; the circadian clock can operate without driving circadian oscillations in *Hes1* and *Bmi1*, and the clock mechanism is not significantly disrupted by any non-rhythmic or ultradian oscillations in the stem cell genes. Because our data acquisition was performed every six hours, the shortest period we could have detected in oscillations of *Hes-1 and Bmi-1* expression was about 12 hours according to the well-known Nyquist-Shannon theorem. Additional work on potential coupling between stem cell genes and the circadian oscillator should also consider sampling at shorter intervals, along with additional replicates at each time point.
If the genes that maintain and regulate the stem cell state of CSCs are oscillating independent of the circadian oscillators in tumors, then additional treatment strategies could be examined. For example, we could conclude that these critical genes are not making use of circadian rhythms in the body to provide them with an advantage in cell growth or differentiation. Therefore, efforts to synchronize oscillators within the circadian timing system to improve patient outcome during cancer treatments would not be expected to accelerate tumor growth by facilitating timing functions in CSCs. Also, use of chronopharmacological methods to direct therapeutic agents to the CSCs could be performed at times of day that are best tolerated by the non-cancer cells while remaining effective against the rapidly oscillating genes of CSCs.
CHAPTER V: CONCLUSIONS

Based on the studies performed in this dissertation the following conclusions can be drawn:

1) The putative CSCSs in C6 monolayers (Hoechst-negative cells) display many of the characteristics of CSCs.

2) The Hoechst-positive cell population of monolayers has a circadian rhythm in PER2 nuclear localization, but the Hoechst-negative cells do not.

3) Hoechst-negative cells comprise about two thirds of the tumorsphere.

4) Tumorspheres grown in medium without added growth factors (MFM) have more uniform and stable circadian rhythms than ones grown in the other two media (SM and SCM).

5) Added growth factors are not required for circadian rhythms and appear to interfere with circadian rhythms, possibly by excessively stimulating intracellular signaling pathways that can affect the circadian timing mechanism.

6) The mPer2 gene promoter is 66.28\% identical in sequence to hPer2 promoter and can be used as a reporter gene to study circadian oscillations of Per2 gene in human cell lines.

7) We detected circadian rhythms in MCF-7 SM monolayer clusters and SCM tumorpsheres. This finding conflicts with previous reports of MCF-7 SM cell cultures lacking circadian rhythms and raises the question of whether MCF-7 cells actually lack functional circadian rhythms. The investigation of MCF-7 circadian rhythms needs further evaluation.

8) We detected robust circadian oscillation of mPer2 transgene in MCF-7 tumorspheres which are highly enriched with CSCs expressing CD133 and Msi-1 marker. Additionally, we also observed high amplitude circadian oscillations in MCF-7 tumorspheres. This finding points toward an alternative mechanism for clock regulation of stem cell self-renewal and differentiation, which might possibly involve the micro RNAs miR192 and miR141.
9) Using a four-point validation approach for in silico identification of the E-box (USF and AHR) elements we identified the cancer stem cell candidate genes \textit{Hes1} and \textit{Bmi1} as potentially under clock control.

10) Two genes that help to maintain stemness display ultradian expression similar to that seen in undifferentiated cells, but they also show evidence of circadian regulation.
CHAPTER VI: FUTURE DIRECTIONS

Future question to be addressed for C6 cancer stem cell study

Using fluorescence microscopy to detect \textit{mPer2} nuclear localization we detected that Hoechst-negative cells of C6 monolayer do not exhibit circadian rhythms. However, this finding needs to be tested with a more reliable approach using single cell bioluminescence study of \textit{mPer2::mPer2::luc} transgene in Hoechst-negative cells.

Additionally, the C6 tumorsphere rhythms that we recorded in our bioluminescence assay could possibly represent only the rhythmic cells present on the periphery of the spheres. Because our system cannot distinguish bioluminescence signal of the periphery cells and the cells present towards the core of the sphere it is hard to tell if the Hoechst-negative cells present inside the sphere are rhythmic. However, since MFM spheres mostly consist of Hoechst-negative cells and exhibit more robust rhythms it is possible the Hoechst-negative cells are rhythmic within the spheres. Either way it is important to study rhythms of single Hoechst-negative cells of the spheres to make an appropriate comparison between the Hoechst-negative cells of the monolayer and the spheres.

Finally, because we observed the Hoechst-negative population enriched in MFM spheres, addressing the clock regulation of cancer stem cell genes in these spheres will be an important question to be addressed.

Because MFM spheres consist of mostly Hoechst-negative cells, it indicates that these cells may appear to be more resistant to chemotherapeutic drugs commonly used in clinics today. It would be important to test whether this is true. If true, this system could be used to test
efficacy of newly synthesized chemotherapy reagents for targeting ABCG2/BCRP-overexpressing CSCs.

**Future question to be addressed for MCF7 cancer stem cell study**

In our study we report the finding that MCF-7 cells are rhythmic as monolayers and spheres in SM and SCM medium, respectively. SCM spheres of MCF-7 cells exhibit larger amplitude in comparison to the MCF-7 cells growing in a differentiating environment. It will be important to further evaluate whether the observed difference in the amplitude has any significance to CSC self-renewal and proliferation. We have proposed a model involving micro RNA regulation of the amplitude of clock gene rhythms. Testing this model may provide insight into previously unknown regulatory mechanisms of the clock and cancer stemness.
CHAPTER VII: ISSUES TO BE ADDRESSED IN CANCER STEM CELL INVESTIGATION

METHODS

Circadian disruption has been long implicated in many cancer types. Many but not all cancer types have been shown to exhibit circadian rhythms. With emerging evidence of CSC involvement in tumor recurrence and drug resistance it has become imperative to study the different aspects of mechanisms at the molecular level that impart the resistance and differentiation capacity in CSCs. Recent studies have shown that a tumor’s molecular signature may be influenced by its microenvironment and the surrounding stromal tissue including the circadian oscillations. Various techniques are in place that enables us to identify, isolate, and study the resistance and metastatic capability of CSCs within tumor tissues, both in vitro and in vivo. However, the output of the techniques used will strictly depend on a number of factors, but mainly how the cells were cultured; markers used for cancer stem cell identification; methods for cancer stem cell isolation; and how the sample was processed to obtain the starting material for use in a given technique. Each of these factors will influence the outcome of studies involving CSCs. This chapter discusses some of these factors in critical detail.

**Culturing cancer stem cells from established tumor lines**

**Culture medium.**

Many of the established tumor cell lines are regularly used in labs to study the cancer stem cells. It is well known that most if not all tumor cell lines consist of a heterogenic population. Depending on the question being addressed one can culture these cells in regular medium containing serum. Exact composition of serum in not well known, but definitely
contains a variety of hormones that support growth, proliferation and differentiation, which is why almost any cell grows well in the presence of serum. To propagate mostly stem-like populations in tumor cell lines a special medium is employed called the stem cell medium (SCM). General composition of SCM varies from lab to lab, but commercially available SCM commonly consists of the following ingredients in addition to their proprietary reagents which are usually unknown to the user who must rely on the word of the supplier that those reagents strictly support stem cell growth.

Supplement for DMEM medium contents (for example Neurocult from Life Technologies)

- 0.9% glucose
- 4 mM L-glutamine
- 25 μg/mL insulin
- 100 μg/mL transferrin
- 20 nM progesterone
- 15 μM putrescine
- 30 nM selenite

With the following additional mitogen supplements

- 20 ng/mL recombinant human Epidermal Growth Factor
- 100 ng/mL recombinant human Platelet-Derived Growth Factor-AA/AB/BB
- 20 ng/mL recombinant human Fibroblast Growth Factor 2
- 2 μg/mL heparan sulfate

Using the SCM growth medium described above one can obtain robust growth of the stem cells in the form of sphere or spheroid bodies. However, the underlying problem of the medium is elevated when a question of gene expression is being addressed in these cells. Progesterone and insulin are known to promote expression of several genes that aid in cancer cell propagation and development. In normal physiology endocrine secretion of sex hormones is required for specific developmental phases, however, whether they are required for all CSCs of all tumor types is not well established and should be questioned critically. Up-regulation of EGFR, FGFR and PDGFR is a well-established fact in many tumors particularly in glioma and breast cancers. Thus, using them as medium ingredients to propagate CSCs is a justified approach.

For circadian studies of the spheroid cells in particular, we have shown that use of many growth factors can interfere with exhibition of circadian oscillations. When similar studies were performed with no exogenous growth factors we obtained smoother and robust circadian signal output. Also, the cells of the spheres consist of stem populations, and a majority of these cell types from different tumors are known to secrete growth factors into the microenvironment which then accumulate in the growth medium over time (medium conditioning). The growth factor released from time to time in the environment should be adequate for stimulation of a stem population to replenish it over time. Thus, when studying circadian rhythms, use of no growth factors or minimal growth factors is highly recommended for occurrence of true circadian signal output.
Use of low-adherent tissue culture plate.

Tissue culture plates with low adherence capacity have been commonly used in labs that propagates CSCs as tumorspheres. The rationale being that when tumor cells are cultured in a 3-D environment they are forced to be reprogrammed to stem-like cells. However, when cultured in a regular tissue culture dish they tend to remain differentiated due to adhering forces that act on the cells. One needs to approach this view very cautiously and ask what is it really happening to cells when cultured in a low attachment environment.

To address this issue we cultured C6 and MCF7 cells in both low attachment and regular tissue culture dishes with attachment factors in SM or SCM. Below is the table that describes the effect from the growth environment on the formation of spheres.
<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Type of Tissue Culture dish</th>
<th>SM medium</th>
<th>SCM Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low-attachment Dish</td>
<td>With regular attachment factors</td>
<td>Low-attachment Dish</td>
</tr>
<tr>
<td>C6 Rat Glioma</td>
<td>Only Spheres</td>
<td>Less spheres (only floating) + mostly adhered and differentiating cell</td>
<td>Only Spheres</td>
</tr>
<tr>
<td>MCF7 Human Breast cancer</td>
<td>Only Spheres</td>
<td>Less spheres (only floating) + mostly adhered and differentiating cell</td>
<td>Only Spheres</td>
</tr>
</tbody>
</table>

Table 4: Analysis of tumorsphere generation in low attachment dish vs. tissue culture dish with attachment factors.
Surprisingly, when the cells were cultured in a differentiating environment (serum containing medium) in low attachment dishes only tumor sphere formation was observed, suggesting that even differentiated cells, due to lack of attachment factors aggregated to form tumor spheres. These tumorspheres were indistinguishable from the tumor spheres formed by these cells in stem cell medium. Interestingly, when the cells were cultured in regular tissue culture dish with attachment factors in stem cell medium, we observed spheres that would form from the monolayer and eventually detach and float in the medium leaving behind the partially differentiated cells that would remain attached on the surface. We observed this phenomenon for both C6 and MCF7 cells. Based on this observation we theorized that on a regular tissue culture dish in SCM only least differentiated stem cells would form tumor spheres and in the presence of adhering forces the partially differentiated cells, would remain attached to the surface and propagate as progenitor cells or further differentiate to produce committed lineages.

Based on our studies and observation of culturing cancer stem cells in SCM medium and on low attachment dishes, we propose that studies on CSCs should critically re-consider what different growth factors are to be used and what type of tissue culture dishes should be employed in culturing these cells. As much as culturing cancer stem cells has been a problem, this view of ours needs to be further evaluated and tested in more controlled experiments.

Markers for identifying cancer stem cells

Several markers for the identification of the CSCs are now identified and published and routinely used in research studies conducted across the globe. A decision to use specific markers for CSCs has been a long-standing debate and is heavily criticized by the scientific community.
The reason for such a discrepancy in use of CSC markers is that if there are four different markers used in the identification of the cancer stem cells in the culture not all four are expressed by any cell. There will be cells that would express one or may be two of the markers. The possible reason underlying this problem is that since the cancer line consists of a heterogeneous population and the stem cells may be at different stages of their differentiation and depending upon which stage of differentiation they are in they may express a specific set of markers. Thus, to make the identification of the CSC marker more reliable one needs to test for the expression of more than three or four different markers in the stem cells. More importantly, CSCs in different tumors exhibit different sets of markers. Thus, a set of markers needs to be defined for CSCs of each tumor type that labs across the globe could use for the identification of stem like cells in their primary tumors or tumor cell lines, making research studies on CSCs more universally consistent.

Based on our understanding of the origin of CSCs in making tumors one needs to consider that stem cells can originate from multipotent stem cells or from progenitor stem cells. Depending upon their origin the CSCs will express markers that would correlate with either multipotent or progenitor stem cells. Primary tumors used to establish permanent secondary cell lines should be evaluated for their origin of CSCs by testing expression of arrays of markers under propagating conditions and determine the population based on marker profile. An estimate can be made about which set of markers are most consistently exhibited by a subpopulation of cells. Based on this estimate one can roughly determine if the tumor initiating cells originated from multipotent or a progenitor cells. Further, a specific set of markers should be designated for the identification of cancer stem cells in that cell line.
Extraction and isolation of total RNA from tumor spheres

Gene expression profile in cancer stem cells is currently the most demanding science in the field of cancer biology. Labs use various defined methods to study gene expression profiles. One of the most reliable ways is to use a luciferase reporter system driven by the promoter of the gene of interest and create a transgenic cellular system to record the expression of a gene of interest in real time. Though this method has advantages over other methods in that it provides information on gene expression as the cells grow over time, it comes with certain disadvantages. It is tedious to create a reporter system for the expression study of every single gene. Importantly, creating a transgenic cell line requires alteration of the genome by the insertion of the transgene construct. Such an insertion can cause the cells to transform from their original property. To overcome this problem an alternative approach of measuring relative mRNA levels has become a favorite approach. If carefully designed, real-time PCR studies can be informative. Especially from the circadian studies point of view, it allows us to look at a variety of clock-controlled genes and is less time consuming when compared to generation of a reporter system.

One of the problems with this approach is that there is no standard method for isolating total RNA from tumorspheres. It is known that the 3-D structure of the tumorspheres is maintained by a hardy extracellular matrix. Conventional RNA isolation kits and methods used for extracting total RNA from tissue culture cells provide very little yield. This limitation is probably because these kits do not have any lytic reagents that will dissociate the spheres completely. If one tries to trypsinize the sphere to isolate the cells, there is more loss of the peripheral cells due to longer incubation time in trypsin. In our hands, physically dissociating sphere cells worked best. Also, we have observed that kits that provide purification columns for isolating RNA from as few as 10 cells are the best columns to use. Determining the number of
spheres to be used for RNA isolation is of particular importance due to their heterogeneity. Standard kits using only 20-30 spheres to extract the total RNA do not provide higher yield. From a circadian point of view it is necessary to isolate RNA from as few spheres as possible to eliminate any heterogeneous expression that may be included from other spheres. An alternative strategy may be to try single-cell isolation kits to isolate total RNA from single spheres.
REFERENCES


76. Lee, J., et al., Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell, 2006. 9(5): p. 391-403.


## APPENDIX A: LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>H33</td>
<td>Hoechst 33342</td>
</tr>
<tr>
<td>CCG</td>
<td>Clock controlled genes</td>
</tr>
<tr>
<td>BLI</td>
<td>Bioluminescence imaging</td>
</tr>
<tr>
<td>SM</td>
<td>Serum medium</td>
</tr>
<tr>
<td>SCM</td>
<td>Stem Cell Medium</td>
</tr>
<tr>
<td>MFM</td>
<td>Mitogen free medium</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>EGF</td>
<td>Endothelial growth factor</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>ADU</td>
<td>Analog digitizer units</td>
</tr>
<tr>
<td>PER</td>
<td>Period</td>
</tr>
<tr>
<td>Bmal1</td>
<td>Brain muscle and ARNT like 1</td>
</tr>
<tr>
<td>CRY</td>
<td>Cryptochrome</td>
</tr>
<tr>
<td>Hes-1</td>
<td>Hairy enhancer of split 1</td>
</tr>
<tr>
<td>Bmi-1</td>
<td>B-cell specific MMLV integration site</td>
</tr>
<tr>
<td>MMLV</td>
<td>Moloney murine leukemia virus</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalo virus</td>
</tr>
<tr>
<td>H3S10-p</td>
<td>Phosphorylated serine 10 on Histone 3 tail</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>Msi-1</td>
<td>Musashi 1</td>
</tr>
<tr>
<td>Olig2</td>
<td>Oligodendrocyte transcription factor</td>
</tr>
<tr>
<td>CD133</td>
<td>Prominin</td>
</tr>
<tr>
<td>SOX2</td>
<td>Sex determining region Y-box 2</td>
</tr>
<tr>
<td>PWM</td>
<td>Positional weight matrix</td>
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</tbody>
</table>