Circadian disruption induced by tumor development in a murine model of melanoma

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Abstract

The circadian system induces oscillations in most physiological variables, with periods close to 24 hours. Dysfunctions in clock-controlled body functions, such as sleep disorders, as well as deregulation of clock gene expression or glucocorticoid levels have been observed in cancer patients. Moreover, these disorders have been associated with a poor prognosis or worse response to treatment. This work explored the circadian rhythms at behavioral and molecular levels in a murine melanoma model induced by subcutaneous inoculation of B16 tumoral cells. We observed that the presence of the tumors induced a decrease in the sturdiness of the locomotor activity rhythms and in the amount of nighttime activity, together with a delay in the acrophase and in the activity onset. Moreover, these differences were more marked when the tumor size was larger than in the initial stages of the tumorigenesis protocol. In addition, serum glucocorticoids, which exhibit strong clock-controlled rhythms, lost their circadian patterns. Similarly, the rhythmic expression of the clock genes *Bmal1* and *Cry1* in the hypothalamic Suprachiasmatic Nuclei (SCN) were also disrupted in mice carrying tumors. Altogether, these results suggest that tumor-secreted molecules (i.e., tumor macroenvironment) could modulate the function of the central circadian pacemaker (SCN). This could account for the worsening of the peripheral biological rhythms such as locomotor activity or serum glucocorticoids. Since disruption of the circadian rhythms might accelerate tumorigenesis, monitoring circadian patterns in cancer patients could offer a new tool to get a better prognosis for this disease.

Keywords: Circadian rhythms; tumor growth, Suprachiasmatic Nuclei, SCN, clock genes, glucocorticoids.

Introduction

Daily environmental changes have imposed a selective pressure for life driving the development of circadian clock mechanisms that generate rhythms in physiological and behavioral variables (e.g. body temperature, hormonal secretion, sleep, locomotor activity, etc.), capable of entrainment to external cues. In mammals, the central clock resides in the hypothalamic Suprachiasmatic Nuclei (SCN), and the principal signal that adjusts its activity is the light-dark (LD) cycle. Circadian rhythms are governed by a molecular machinery which maintains rhythmic precision within cells and synchrony between central and peripheral clocks (e.g. liver, lung, spleen, among others) (Golombek et al., 2013). The molecular mechanism of the circadian clock arises from negative transcriptional feedback, which generates oscillations with periods close to 24 hours. The core loop includes the positive elements Clock and Bmal1, inducing the expression of the negative elements Per1-3 and Cry1-2, which, in turn, repress the transcriptional activity of the positive elements in a process where compartmentalization, shuttling, and posttranslational events determine the pace of the circadian clock. This cell-autonomous pacemaker mechanism has been found in almost every cell in the body (Chen et al., 2018).

Bidirectional interactions between tumor development and the circadian system have been under intensive study in recent years. Dysfunction in clock-controlled physiologic variables has been observed in cancer patients. The prevalence of sleep-related disturbances (including poor quality of sleep, insomnia, daytime sleepiness or fatigue) in different types of cancer ranges from 45% to 80% (Chen et al., 2015; Halle et al., 2017; Bulbul et al., 2018). These disorders were also observed in cancer survivors (Harrington et al., 2010). In addition, in patients suffering colorectal

metastatic cancer, the activity circadian patterns correlate with survival, quality of life, physical and social functioning, fatigue and appetite loss (Mormont et al., 2000; Innominato et al., 2009). However, the causes of these rhythm disruptions in cancer are currently unknown.

On the other hand, there is an increasing body of evidence showing an abnormal expression of clock genes associated with the development and progress of malignant tumors in humans (Qiu et al., 2019a; Qiu et al., 2019b). In particular, *Per1* and *Cry1* gene expression is downregulated in endocrine tumor tissues, suggesting a suppression of the peripheral clock system. In contrast, in testicular tumors the heterodimer Clock/Bmal1 was also found to be downregulated, whereas in ovarian cancer data suggest a preserved clock system [reviewed in (Angelousi et al., 2019)]. However, the specific mechanisms that lead to these differences in the expression of the clock genes remain unclear. Furthermore, a quantitative analysis of the expression levels of core clock genes revealed significant differences between responders and non-responders to neoadjuvant chemoradiation therapy, suggesting that clock genes could also serve as potential biomarkers to predict patient responses (Lu et al., 2015). Nevertheless, it is still not clear whether clock gene disruption is the cause or the consequence of tumorigenesis. There is data showing that clock gene-deficient mice have a higher tumor incidence (Lee et al., 2010), but the tumoral effect on clock gene expression remains to be explored. These facts also suggest that the correct synchronization of circadian rhythms could be a significant tool to improve the prognosis in cancer patients.

The central clock is capable to adapt to peripheral fluctuations as a compensatory mechanism. For instance, cytokines and chemokines modulate the

activity of the SCN (Paladino et al., 2014; Duhart et al., 2016), and feeding time schedule changes circadian gene expression in the liver (Stokkan et al., 2001; Vollmers et al., 2009; Eckel-Mahan et al., 2013). In this regard, there are severe changes in the livers of a lung adenocarcinoma murine model, including alterations in pro-inflammatory response, insulin, glucose and lipid metabolism (Masri et al., 2016). Tumor-derived metabolites and cytokines constitute the so-called tumor macroenvironment (Al-Zoughbi et al., 2014); however, their specific consequences on the circadian system remain elusive. The knowledge of the tumor-induced circadian changes could be important not only to improve the quality of life of cancer patients, but also to design more efficient chronotherapies depending on the synchronization state of the patient. This is even more relevant since circadian disorders favour the development and progression of tumors (Aiello et al., 2020), thus, tumor-induced circadian disruption could start a vicious circle that worsens the prognosis of cancer patients. Here we provide evidence of circadian disruption in a murine model of melanoma.

Materials and methods

Animals

Adult (2-month old) C57bl/6J WT male mice (*Mus musculus*) were raised in our colony. Mice were housed under a 12:12-h LD photoperiod (with lights on at 7:00 h and lights off at 19:00 h) with food and water ad libitum for at least 2 weeks before entering into experimental conditions. All animal experiments were carried out in accordance with international ethical standards for the care and use of laboratory animals (Portaluppi et al., 2010).

Experimental tumorigenesis protocol

The melanoma B16F0 cell line, kindly provided by the Oncology Laboratory of the National University of Quilmes, was maintained in culture medium (DMEM, GIBCO) containing 5% of fetal bovine serum (FBS) at 37°C and 5% CO_2. To induce tumorigenesis, 30,000 cells were subcutaneously injected in the right flank of the mice. Animals were manually palpated 3 times a week and the day of tumor detection was recorded (latency to approximately 1 mm^3). Next, the tumor size was measured with a caliper three times a week. Mice were anesthetized using 5% isoflurane and sacrificed when the tumor reached 1500-2000 mm^3.

Behavioral data analysis

Mice were housed in individual cages and general activity was detected by infrared motions sensors connected to a computer interface that records activity counts every five minutes for posterior time-series analysis (Archron, Buenos Aires, Argentina). The general activity was recorded and divided into three timeframes: Basal: 10 days prior to tumoral cells inoculation, Latency: from cell inoculation to tumor detection (approximately 1 mm^3) and Growth: tumor detection to the end point of the tumorigenic protocol (tumor size of 1500-2000 mm^3). , a

The activity pattern analysis, waveforms, periodograms and cosinor analysis were performed using the El Temps (version 1.219, University of Barcelona) and autocorrelation analysis using the Matlab software. Waveforms show the average activity of the successive days in each time point evaluated within the LD cycle. Individual waveforms were performed to quantify the amount of activity during the whole day or during light or dark phases as the area under the curve occurring within the corresponding interval. Phase angle (**φ**) was calculated as the difference in minutes between the activity onset and lights off, and positive values indicate a delay in the

activity onset which is showed as minutes after lights off. The onset time was identified in the waveform as the moment when the activity curve crossed, and was higher than the mean line. A 24-h cosinor adjustment was performed in order to obtain amplitude and acrophase values. Acrophases, which are shown as minutes after lights off, were entered into Rayleigh z tests to evaluate the clustering inside of each experimental group and facilitate display of the position of the mean vector. All groups showed statistically significant grouping of their acrophase values. Data corresponding to the power of the period (%V) was obtained from the Bonferroni-corrected Sokolove-Bushell (SB) periodograms as the percentage of the variance (Sokolove et al., 1978). Periodograms were evaluated from 1200 minutes (20 h) to 1600 minutes (26:40 h). In all animals, the period was statistically significant (Chi-Squared test) and the mean period obtained was 24±0.15 h. Rhythmicity index (RI) and interday variability (IV) were calculated using autocorrelation analysis, plotting the correlation coefficient on the vertical axis versus time-lags plotted on the horizontal axis. A correlation coefficient was calculated for the activity record against itself for each lag. The RI was obtained as the height of the third peak of the autocorrelogram. The IV evaluated the stability of this pattern in the successive cycles (days). Time is expressed as *Zeitgeber* Time (ZT), with ZT0 defined as the time of lights on and ZT12 as the time of lights off in LD conditions.

RNA extraction and real-time PCR

Tissues corresponding to SCN regions were carefully dissected from mice. Total RNA was isolated using 100 µl of TRIzol reagent (Life Technologies) according to the manufacturer's instructions and 1 µl of LPA (Linear Polyacrylamide; 1 µg/µl) was added in the EtOH precipitation step to improve the purification efficiency. RNA

solutions were quantified using a NanoDrop1000 equipment (Thermo Scientific) and their integrity was evaluated by electrophoresis. cDNA was synthesized using 200 ng of total RNA, oligo(dT) primers and the SuperScript II™ First-Strand Synthesis System (Invitrogen). Gene amplification was performed on a Stratagene Mx3000P real-time PCR instrument (Agilent Technologies), using 10 µl of final reaction volume containing 0.5 µl of cDNA as template, 1X of the FastStart Universal SYBR® Green Master (Roche, 04913850001) and the corresponding primers (Suppl. Table 1) in a final concentration of 300-400 nM. The cDNA template was amplified in triplicate, with the following conditions: 95ºC for 2 min, followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min. Then the melting curve was obtained between 60 and 95ºC. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with *Hprt* gene used as reference.

Glucocorticoid determination

Blood samples were obtained by cardiac puncture at the endpoint of the tumor protocol and centrifuged at 6000 rpm for 10 min to obtain the serum. 1/10 dilutions of these samples were extracted with Dichloromethane. Next, the serum levels of Corticosterone were measured by Radioimmunoassay (RIA) in the High Level of Corticosterone Determination Technological Service of the School of Medicine of the Buenos Aires University. A standard curve was constructed between 31 and 1000 µg/100 µl.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Behavioral data was analyzed using repeated measures ANOVA test and differences between time points were evaluated using the Kruskal Wallis test (non-parametric ANOVA). Linear regression was applied for the correlation analyses between behavioral data and the corresponding R^2 was obtained. P values less than 0.05 were considered to be

statistically significant. GraphPad Prism7 and Infostat were used to perform these analyses.

Results

The presence of tumors modifies circadian parameters of locomotor activity

In order to evaluate the effect of tumorigenesis on the circadian system, mice maintained under LD condition were inoculated subcutaneously with 30,000 B16F0 melanoma cells. Tumors took 12$\pm$0.66 days to be detected (time of Latency to approximately 1 mm^3) and 12.9$\pm$0.39 days since detection to reach the end point (1500-2000 mm^3 approximately), thus, the total tumorigenic process took 24.9$\pm$0.85 days (data not shown). We detected tumoral growth in 90% of the inoculated animals. In order to evaluate the locomotor rhythm, the activity recording of mice inoculated with tumor cells or vehicle was divided into three timeframes: Basal: 10 days prior to cell inoculation, Latency: from cell inoculation to tumor detection and Growth: tumor detection to the end point.

As shown in Figure 1A, animals carrying tumors showed a reduction in the amount of the total activity ($p<0.0001$), which deepened throughout the tumorigenic process (Basal vs. Growth: $p<0.0001$; Latency vs. Growth: $p<0.01$). This reduction was due to a decrease in nighttime activity (Fig. 1B. $p<0.0001$), which was more profound over time (Basal vs. Latency: $p<0.01$; Basal vs. Growth: $p<0.0001$; Latency vs. Growth: $p<0.05$), since there was no statistical difference in the amount of daytime activity (Fig. 1C). In addition, control animals did not show differences in the amount of activity (Fig. 1 D-F). Figures 2A-D show representative actograms and waveforms of mice carrying tumors and controls. The representative waveform from tumor-bearing mice, which

superimposes the waveforms of the different stages of the disease, evidences the decrease of the activity counts with the advance of the tumorigenic protocol (Fig. 2C).

Next, a cosinor adjustment to a 24-h period of locomotor activity recording was performed to evaluate circadian parameters as amplitude and acrophase. As shown in Figure 3A, the amplitude decreased in mice carrying tumors ($p<0.0001$), both in Latency and Growth stages of the disease (Basal vs. Latency: $p<0.01$; Basal vs. Growth: $p<0.0001$). This result is in line with the decrease of the nighttime activity shown in Figure 1. The acrophase (peak of the activity rhythm) showed a delay in tumor-bearing mice (Fig. 3B; $p<0.0001$). In Basal conditions, the peak of the activity was observed 348.7 ± 11.6 minutes after lights off, while in the Latency and Growth period its value was 434.6 ± 22.51 and 449.4 ± 23.8 minutes after lights off, respectively (Basal vs. Latency: $p<0.001$; Basal vs. Growth: $p<0.0001$). This delay is also observed in the superimposed Rayleigh z graphs as the shift in the direction of the mean vector obtained in each disease stage (Fig. 3C). Representative cosine adjustment curves show the important decrease in the amplitude and the delay of the acrophase throughout the tumorigenic protocol in a tumor-bearing mouse, which is not observable in the control mouse (Fig. 2E-F). Accordingly, the onset of activity was also delayed since the phase angle increased in mice carrying tumors (Fig. 3D; $p=0.0017$). This difference was mainly observed in the tumor growth period (Basal vs. Growth: $p<0.001$; Latency vs. Growth: $p<0.05$). Again, these parameters did not show statistical differences in control mice (Fig. 3E-H).

Additionally, the sturdiness of the activity rhythms decreased during the tumorigenic protocol (Fig. 4A-B; A: $p=0.0002$, B: $p<0.0001$). Both the power of the periodogram and the rhythmicity index were lower in the animals carrying tumors than

in their basal conditions (A: Basal vs. Growth: p<0.0001; Latency vs. Growth: p<0.0001; B: Basal vs. Latency: p<0.01; Basal vs. Growth: p<0.0001). On the other hand, the interday variability did not show differences between the stages of the tumoral growth (Fig. 4C). Again, control animals did not show any of the changes observed in mice carrying tumors (Fig. 4D-F). The superimposed periodograms show the decrease in their amplitude (%V) throughout the tumorigenic protocol in a representative mouse that carried the tumor, but not in the control mouse (Fig. 2G, H).

Next, correlation analyses were performed with parameters obtained from the Growth period of tumor-bearing mice. Statistically significant correlations were obtained for several parameters of the activity recording (Suppl. Fig. 1). Between them, the interday variability (IV) correlated negatively with the amount of nighttime activity (p=0.001; Suppl. Fig.1A) and the amplitude (p=0.002; Suppl. Fig.1B). In addition, the power of the periodogram (%V) positively correlated with the rhythmicity index (RI; p=0.037; Suppl. Fig.1C), with the amplitude (p=0.014; Suppl. Fig.1D) and with the amount of nighttime activity (p=0.0007; Suppl. Fig.1E). Finally, the amplitude negatively correlated with the phase angle (p=0.035; Suppl. Fig.1F). These data suggest that the decrease of the activity is accompanied by the worsening of the parameters related to the robustness of the activity rhythms (IV, %V and RI). As expected, the amplitude positively correlated with the amount of nighttime activity ($R^2=0.90$, p<0.0001, data not shown). Since these analyses take into account the parameters obtained for the same mouse, our results confirm the data previously shown and reinforce the impact of the tumor presence on the activity rhythm parameters.

Since mice are subject to a very strong *zeitgeber*, the LD cycle, and the tumorigenesis protocol lasts only 25 days (approximately), These data suggest an

important effect of tumor presence on the circadian system function.

Inhibition of peripheral glucocorticoids rhythms in tumor-bearing mice

To assess whether other peripheral rhythms were affected by the tumorigenic process, we evaluated the serum glucocorticoids rhythm, an important output from the central clock, in mice carrying tumors and controls maintained under LD conditions. This stress-related hormone participates in multiple body functions, like homeostatic, metabolic and immune mechanisms (Russell et al., 2019), and could be relevant during cancer disease. As shown in Figure 5A, control mice exhibited a rhythm of serum corticosterone with maximum levels at the beginning of the night (ZT12-15; p=0.0014) (as previously shown by Filipski et al., 2004; Gong et al., 2015; Van Dycke et al., 2015). Importantly, when we evaluated mice carrying tumors, we did not observe any differences between time points (Fig. 5B). The lack of rhythmic secretion of glucocorticoids indicates that the tumor presence also affects peripheral clocks and suggests that the whole circadian system could be malfunctioning.

Tumorigenesis-induced modulation of clock gene expression in SCN

In order to elucidate whether the observed defects in the peripheral clocks could be related with the function of the central clock, the mRNA levels of the clock genes *Bmal1* and *Cry1* were evaluated in the SCN at different time points in mice carrying tumors and controls maintained under LD conditions. As expected, in control mice, *Bmal1* levels peaked at the end of the night and the beginning of the day (ZT21-0; Fig. 6A; p=0.02) (Van Dycke et al., 2015), while *Cry1* peaked at the end of the day (ZT9; Fig. 6B) (Bonaconsa et al., 2014). Interestingly, in tumor-bearing mice, no significant differences were observed between different time points, for both *Bmal1* and *Cry1* genes (Fig. 6C, D).

Taken together, these results indicate that the tumorigenic process induces a strong modulatory effect on the circadian system both at central and peripheral levels. The loss of rhythms of clock genes in the SCN indicate a defect in the central clock function and could explain the worsened rhythmic parameters observed in the locomotor activity and the glucocorticoid rhythms.

Discussion

Here we show that the tumor presence can negatively modulate circadian rhythms. In particular, we were able to determine several deficits in locomotor activity rhythms, together with the disruption of rhythms in serum glucocorticoid levels. Importantly, this worsened rhythmicity was associated with the deregulation of the daily rhythms of the clock genes in the SCN. These data suggest that molecules secreted by the tumor macroenvironment modulate the function of the central circadian clock, and thus, modify its outputs, such as rhythms in locomotor activity and glucocorticoid serum levels. In addition, using the same melanoma model, we have previously reported a similar rhythmic pattern of these clock genes in the liver and the disruption of the daily pattern of immune cells in the spleen of tumor-bearing mice in comparison with controls (Aiello et al., 2020). Notwithstanding, the deregulation of the circadian system induced by the tumor macroenvironment is not complete as rhythmic patterns persist in animals.

The impairment of locomotor activity rhythms includes a reduction in the amount of nighttime activity, its amplitude and robustness (%V, RI and IV), together with a phase delay of this pattern (acrophase and phase angle). Both the power of the periodogram and the rhythmicity index show the sturdiness of the 24-hours activity rhythms calculated by different ways, while the interday variability describes the

similitude between the successive days taking into account the activity episodes during the day and the rest episodes during the night. The observed changes of these parameters throughout the tumorigenic protocol indicate a worsening of the locomotor circadian rhythms. In addition, the delay observed in both the acrophase and the activity onset also showed the modulation of the circadian parameters in mice carrying tumors. These data suggest a functional modification of the central clock which could be related to the deregulation of the rhythmic expression of the clock genes *Bmal1* and *Cry1* in the SCN. However, since the activity patterns continue to be rhythmic (all animals showed a significant 24-h period), even if weaker, it is possible that the clock molecular machinery sustains a differential control of overt rhythms. Another possibility is that the activity pattern was masked by the LD cycle, and therefore responds directly to light without being regulated by the SCN. This resembles the phenotype of *Cry1*-deficient mice, where the locomotor activity is still rhythmic under LD, but not under free-running (DD), conditions (Vitaterna et al., 1999) and Per2 continues to be rhythmic in SCN explants (measured by luciferase activity) (Liu et al., 2007); moreover, the length of the free-running activity period depends on the level and site-specific phosphorylation of Cry1 protein (Ode et al., 2017). Interestingly, it was recently reported that Cry1 confers robustness, but not rhythmicity, to the circadian system (Putker et al., 2021). Similarly, Bmal1-deficient mice also keep the synchrony of their locomotor activity in LD and become arrhythmic under DD conditions. Again, in line with our results, these mutants showed a decrease in the total activity counts together with an increase in the percentage of the activity occurring during the day and an advance in the phase angle under LD conditions (Bunger et al., 2000).

In spite of the evidence regarding sleep and activity disorders in cancer patients (Chen et al., 2015; Halle et al., 2017; Bulbul et al., 2018), there are very few works

exploring the impact of tumoral presence on the activity circadian pattern in animal models. Mice subject to an experimental hepatocarcinoma model showed a reduction in the amplitude of the locomotor activity and temperature rhythms (Mteyrek et al., 2017) and, in a mammary tumor model, an increase in the phase angle and in the amount of day activity, and a decrease of the night activity counts were reported (Sullivan et al., 2019). These data suggest a direct impact of the tumor presence on the circadian system. We speculated that the decrease of the total activity was not directly due to a difficulty in movement caused by the presence of a large subcutaneous tumor since, for both activity counts and circadian parameters, we show changes in the Latency period in which the tumor volume is less than 1 mm^3. Moreover, in the mentioned mammary tumor model the size of the tumors are similar to the ones used here and similar values of whole day activity both for general and wheel-running recordings were observed (Sullivan et al., 2019). Furthermore, in the hepatocarcinoma model, which generates small tumor nodules observable in the liver by histopathology, a reduction in total activity counts was induced (Mteyrek et al., 2017). On the other hand, in cancer patients, robust rest-activity circadian rhythms and adequate light intensity physical activity improve sleep quality (Chen et al., 2015) indicating the relevance of chronobiology-driven treatments.

We also observed the loss of serum corticosteorid rhythms in mice carrying tumors. This result is in line with the alteration in the rhythm of serum cortisol reported in cancer patients [reviewed in (Mazzoccoli et al., 2010; Vitale et al., 2018)]. Interestingly, this pattern was associated with the evolution of the disease, showing a flat pattern in individuals with worst prognosis (Kim et al., 2012). Additionally, the alteration of the glucocorticoid rhythm before treatment was more marked in patients who did not respond to chemotherapy in comparison with those who responded to it

(Lissoni et al., 2008b). On the other hand, mice inoculated with mammary tumors also showed the abolition of corticoid serum rhythms (Sullivan et al., 2019). However, in other experimental models there are reports showing normal corticoid levels in mice carrying tumors (Azpiroz et al., 2008; Ji et al., 2012; O'Connor et al., 2013; Valles et al., 2013). Additionally, an increased level of glucocorticoids was assessed in a metastatic melanoma model (Vegas et al., 2009). However, only one of these works evaluated different time points (Valles et al., 2013) indicating the relevance of studying the complete daily pattern.

Glucocorticoids serve several functions, including the downregulation of the immune system. It is relevant to mention that the alteration of cortisol was accompanied with changes in lymphocyte counts and cytokines levels in cancer patients, suggesting a possible causal relationship with poor prognosis (Lissoni et al., 2008a; Du et al., 2013). Similarly, in other diseases such us obesity or diabetes, disrupted cortisol rhythms were associated with a deregulation in the rhythms of insulin, adiponectin, Interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, leptin, ghrelin and the chemokine CCL-2 [Reviewed in (Vitale et al., 2018)]. Additionally, the impairment of the circadian pattern of glucocorticoid levels induced alterations in emotional, cognitive and metabolic processes [reviewed in (Russell et al., 2019)], which could be related with cancer-related symptoms such as depression or fatigue.

On the other hand, here we report the disruption of the rhythms of clock genes in the SCN. The disruption of *Bmal1* expression in the central clock was recently observed using another melanoma model (de Assis et al., 2018). Several works have shown the modulation of clock gene expression for cancer-related molecules. The levels of c-Myc, an important protein involved in cell proliferation, is upregulated in many tumors. It has

been reported that c-Myc inhibits the expression of *Bmal1* and *Clock* genes in U2OS cells (Shostak et al., 2016b). Moreover, the level of c-Myc inversely correlated with Bmal1, Clock, and Cry1/2 levels in human lymphomas (Shostak et al., 2016b). C-Myc is a transcription factor that binds the genome through E-boxes, the same binding sites of the Bmal1/Clock heterodimer. ChIP-seq analyses revealed that c-Myc is recruited to these DNA-binding sequences suggesting that it could activate transcription of the E-box-controlled negative regulators of the circadian clock (Shostak et al., 2016a). Additionally, other works reported that c-Myc inhibit the expression of *Bmal1* through the upregulation of Rev-Erb-α and Rev-Erb-β, the secondary oscillator loop components that dampen *Bmal1* expression (Altman et al., 2015; Altman et al., 2017). On the other hand, *Bmal1* gene is transcriptionally silenced by promoter CpG hypermethylation in hematologic malignancies, such as diffuse large B-cell lymphoma and acute lymphocytic and myeloid leukemias, impairing the characteristic circadian expression pattern of genes such as c-Myc, among others (Taniguchi et al., 2009). In addition, p53, an important negative regulator of the cell division machinery, downregulates Bmal1/Clock transcriptional activity by blocking their interaction with E-box elements (Miki et al., 2013). On the other hand, the cancer/testis antigen PIWIL2 represses circadian rhythms in osteosarcoma cells by inactivation of GSK-3β. GSK-3β phosphorylate several core-clock proteins, leading to either their degradation (in case of Cry2, Clock and Bmal1) or their increased nuclear translocation (Per2) or stabilization (Rev-Erb-α) (Yin et al., 2006; Sahar et al., 2010; Angelousi et al., 2019). Meanwhile, PIWIL2 can bind E-Box sequences associated with the Bmal1/Clock complex to negatively regulate their transcriptional activity on promoters of clock-controlled genes (Lu et al., 2017). Taken together, these data suggest that tumors have different mechanisms to modulate the circadian system, which should be studied in each specific

situation.

Several important research points arise from the analyses of circadian rhythms during the tumorigenic process. First, in spite of the circadian expression of clock genes in some tumor cell lines in vitro, clock gene expression is generally impaired in most human cancers (Matsu-Ura et al., 2018) and animal models of tumorigenesis (Aiello et al., 2020). Moreover, the number of rhythmic genes is reduced in human cancer samples (Kiessling et al., 2017b; Angelousi et al., 2019) and in immortalized cell lines as compared to normal tissues (Matsu-Ura et al., 2018). In addition, intratumoral injection of dexamethasone in mice not only induced the rhythmic expression of clock genes and a rhythmic pattern of mitosis and apoptosis in B16 melanoma tumors, but also reduced tumor growth rate (Kiessling et al., 2017a). In this context, the deregulated circadian pattern of glucocorticoids reported here could facilitate tumor growth. This suggestion could be relevant since B16 tumors showed a lack of circadian rhythms in clock genes *Cry1* and *Bmal1* (Kiessling et al., 2017a; Aiello et al., 2020).

Second, since the tumor macroenvironment modulates several metabolic and homeostatic processes [reviewed in (Lee et al., 2016)], it is expected to also modify circadian physiology. In fact, circadian rhythms were also altered in normal tissue surrounding tumors (de Assis et al., 2018) and in other healthy tissues both in cancer patients and animal models [reviewed in (Masri et al., 2018)]. In particular, liver circadian homeostasis is disturbed in murine models of lung adenocarcinoma (Masri et al., 2016) and breast cancer (Hojo et al., 2017). Moreover, skin tumors modify the expression of clock and clock-controlled genes in the lung and in the liver (de Assis et al., 2018). Additionally, the serum from lung cancer patients is able to modify the expression of clock genes in cell culture (Chang et al., 2021). In line with this evidence,

here we show a strong modulatory effect of the tumor presence on the rest-activity and glucocorticoids circadian patterns, which could be explained by the alteration of clock genes in SCN. Since cytokines and chemokines, which are normally secreted during the tumorigenesis process by immune cells, can modulate SCN function (Paladino et al., 2014; Duhart et al., 2016), it is possible to hypothesize that these molecules could be implied in the observed results. In addition, not only TNFR1- (receptor of TNF-α) and IL6-deficient mice showed slight, but significant, differences in their activity circadian patterns but also IL-6 deficient mice exhibit an increase in the levels of the clock genes *Cry1*, *Dec2* and *Rev-Eerb-β* in hippocampal tissue (Monje et al., 2017).

Lastly, as previously described, cancer patients suffer several clock-related symptoms suggesting that the modulatory effect of the tumor on the circadian system impacts negatively on their whole physiology. This could be related to both the mentioned immune-related molecules and the glucocorticoid rhythms. In particular, the cytokines IL-1, IL6 and TNF-α induce sickness behavior and depression, including symptoms such as decreased activity, increased and disturbances of the sleep, anorexia, fatigue, loss of interest in usual activities, social withdrawal, listlessness, hyperalgesia, and cognitive dysfunction (Lee et al., 2004). In addition to the cytokines, the chemokine CCL2 and its receptor CCR2 seem to be involved in this phenomenon [reviewed in (D'Mello et al., 2017)]. These molecules not only modulate the activity of the central clock in the SCN, but also in the Paraventricular Nuclei [reviewed in (Gadek-Michalska et al., 2013)], the brain center controlling glucocorticoid secretion, which, as mentioned before, is related with cancer symptoms.

In conclusion, the presence of the tumor significantly disturbs the circadian system. However, more research is needed to further understand the mechanisms

involved in this phenomenon. The evaluation and the improvement of the circadian rhythms in cancer patients could not only improve their quality of life, but also their prognosis and response to treatment, both conventional or chronomodulated.

Disclosure of interest

The authors report no conflict of interest.

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TABLE AND FIGURE LEGENDS

Figure 1. Locomotor activity patterns of tumor-bearing mice. Mean ± SEM of total (A, D), nighttime (B, E) and daytime (C, F) activity of mice subcutaneously inoculated with 30.000 B16F0 melanoma cells (A-C) or vehicle (D-F). The general locomotor activity was recorded with infrared motion sensors. The amount of activity was calculated by waveform analysis, measuring the area under the curve for each interval (day and night). Basal: 10 days prior to tumoral cell inoculation, Latency: from cell inoculation to tumor detection (approximately 1 mm^3) and Growth: tumor detection to the end point (tumor size of 1500-2000 mm^3). Repeated measures ANOVA test: A) $p<0.0001$, B) $p<0.0001$; post hoc test: *$p<0.05$, **$p<0.01$, ****$p<0.0001$. n=18 for mice carrying tumors and n=8 for control mice.

Figure 2. Representative pictures of the locomotor activity analyses. Actograms (A, B), waveforms (C, D), cosinor adjustments (E, F) and periodograms (G, H) obtained from the activity recording of a mouse carrying tumor (A, C, E, G) and a control mouse (B, D, F, H). Basal: days prior to tumor cell inoculation, Latency: from cell inoculation to tumor detection (approximately 1 mm^3) and Growth: tumor detection to the end point (tumor size of 1500-2000 mm^3). Actograms, waveforms and cosinor adjustments are doubly plotted (48 hours) to improve their display and bars above these show the LD cycle (day in white and night in black).

Figure 3. Circadian parameters of activity rhythms in mice carrying tumors. Mean ± SEM of amplitude (A, E) and acrophase (B, F), Rayleigh z test of acrophase values (C, G) and mean ± SEM of phase angle (D, H) obtained from the locomotor activity recordings of mice subcutaneously injected with 30.000 B16F0 cells (A-D) or vehicle (E-H). The general locomotor activity was recorded with infrared motion sensors. Amplitude (A, E) and acrophase (B, F) were obtained from a 24-h cosinor adjust of the activity recordings. Superimposed Rayleigh graphs (C, G) show the grouping and the mean vector obtained from acrophase values of each stage of the tumorigenic protocol. The phase angle (D, H) showed the difference between the activity onset and the light off, and positive values indicate a delay in the activity onset. Basal: 10 days prior to cell inoculation, Latency: from cell inoculation to tumor detection (approximately 1 mm^3)

and Growth: tumor detection to the end point (tumor size of 1500-2000 mm^3). Repeated measures ANOVA test: A) p<0.0001, B) p<0.0001, D) p=0.0007; post hoc test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Rayleigh z test: C) Basal: p=2.87382e-11, Latency: p=0.0004, Growth: p=0.00027; G) Basal: p=0.0001, Latency: p=0.0004, Growth: p=0.0001. n=18 for mice carrying tumors and n=8 for control mice.

Figure 4. Sturdiness of activity rhythms in tumor-bearing mice. Mean ± SEM of the power of the periodogram (A, D), the rhythmicity index (B, E) and the interday variability (C, F) of the activity pattern of mice subcutaneously injected with 30.000 B16F0 cells (A-C) or vehicle (D-F). The general locomotor activity was recorded with infrared motion sensors. The SB periodograms were performed to obtain their percentage of variability which indicates the power of the periodogram (%V). All periodograms result statistically significant. Rhythmicity index and interday variability were obtained from autocorrelation analyses. Basal: 10 days prior to cell inoculation, Latency: from cell inoculation to tumor detection (approximately 1 mm^3) and Growth: tumor detection to the end point (tumor size of 1500-2000 mm^3). Repeated measures ANOVA test: A) p<0.0001, B) p=0.0002; post hoc test: **p<0.01, ****p<0.0001. n=18 for mice carrying tumors and n=8 for control mice.

Figure 5. Rhythms of serum glucocorticoids in mice carrying tumors. Mean ± SEM of serum levels of corticosterona of mice inoculated subcutaneously with vehicle (A) or 30.000 B16F0 cells at endpoint (B) maintained under LD conditions detected by Radioimmunoassay (RIA). A) Kruskall-Wallis test: p=0.0014; post hoc test: **p<0.01 vs. ZT0. n=3-4 per condition and time point.

Figure 6. Rhythms of clock genes in SCN of mice carrying tumors. Mean ± SEM of relative mRNA levels of *Bmal1* (A, C) and *Cry1* (B, D) in SCN of mice inoculated subcutaneously with vehicle (A, B) or 30.000 B16F0 cells at endpoint (C, D) maintained under LD conditions detected by Real-Time PCR. A) Kruskall-Wallis test: p=0.02; post hoc test: p<0.05 for a) ZT0 vs. ZT3, ZT9, ZT12 and ZT15, b) ZT21 vs. ZT3 and ZT9. n=3-4 per condition and time point.

Supplementary Figure 1. Correlation analysis of circadian parameters in tumors-bearing mice. Correlation between interday variability (IV) and nighttime

activity (A), amplitude (B), between power of the periodogram (%V) and rhythmicity index (RI; C), amplitude (D), or nighttime activity (E), and between amplitude and phase angle (H) of data obtained from the Growth period of mice carrying tumors. Linear regression was plotted in each graph. R^2 and p values are shown in the corresponding graphs. n=18.

Table S1. Primers sequences used for real-time PCR.

WORD COUNT: 8,388