Connection between clock gene desynchronization and mitochondrial dysfunction in head and neck squamous cell carcinoma: evaluation of melatonin effects

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Dº César Rodríguez Santana

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Fdo: César Rodríguez Santana

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Fdo: Dra. Germaine Escames Rosa

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César Rodríguez Santana

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ABBREVIATIONS

AANAT: arylalkylamine N-acetyltransferase

ADP: Adenosine diphosphate

AFMK: N1-acetyl-N2-formyl-5-methoxykynurenamine

AMK: N-acetyl-5-methoxykinurenamine

aMT: 5 methoxy-N-acetyltryptamine, melatonin

ASMT: N-acetylserotonin O-methyltransferase

ATP: Adenosine triphosphate

BIM: Bcl-2-interacting mediator of cell death

Bmal1: brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1

CAF: cancer-associated fibroblast

CCG: clock-controlled gene

CHRONO: chip-derived repressor of network oscillator

CI-V: Complex I-V

CK1 ε/δ: cysteine kinase 1 ε/δ

Clock: circadian locomotor output cycles kaput

Cry: chryptochrome

DCs: dendritic cells

Diacetate

DMEM: dulbecco's modified Eagle's medium

DNA: deoxyribonucleic acid

ETS: electron transfer system

FBS: fetal bovine serum

FCCP: trifluoro carbonyl cyanide phenylhydrazone

GABA: gamma-aminobutyric acid

GPx: glutatione peroxidase

GSH: reduced glutathione

HAT: histone acetylase

HDAC: histone deacetylase

HIF-1α: hypoxia-inducible factor-1α

HNC: Head and neck cancer

HNSCC: Head and neck squamous cell carcinoma

HPV: human papillomavirus

IARC: International Agency for Research on Cancer

ipRGCs: photosensitive retinal ganglion cells

MMP9: matrix metalloproteinase 9

MOMP: mitochondrial outer membrane permeabilization

mPTP: mitochondrial permeability transition pore

mtDNA: mitochondrial DNA

mtROS: mitochondrial ROS

NAD+: nicotinamide adenine dinucleotide (oxidated)

NADH: nicotinamide adenine dinucleotide (reducited)

NAMPT: nicotinamide phosphoribosyl transferase

NF-kB: nuclear factor kappa B

NPAS2: neuronal PAS domain protein 2

Nrf2: nuclear factor erythroid 2-related factor 2

OCR: oxygen consumption rate

OSCC: oral squamous cell cancer

OxPhos: oxidative phosphorylation system

PBS: phosphate buffer solution

Per: period

PG: pineal gland

PGC-1α: peroxisome proliferator-activated receptor-gamma coactivator

PI3K: phosphatidylinositol 3-kinases

PKM2: pyruvate kinase M2

PVN: paraventricular nucleus

RET: reverse electron transport

Rev-erbα: reverse strand of protein ERB alpha

Rev-erbα: reverse strand of protein ERB alpha

RNA: ribonucleic acid

RORα: orphan retinoic acid receptor related alpha

RORα: orphan retinoic acid receptor-related alpha

ROS: reactive oxygen species

SCN: suprachiasmatic nucleus

SEM: standard error of the mean

SIRT: sirtuin

SOD: superoxide dismutase

TCGA: the cancer genome atlas

TTFL: transcription–translation feedback loop

VEGF: vascular endothelial growth factor

WHO: World Health Organization

“Dicen que la curiosidad mató al gato, pero
no dicen si lo que descubrió valió la pena”

José Saramago

A mi familia

RESUMEN

Los ritmos circadianos son un sistema regulador, con una periodicidad aproximada de 24 h, que genera cambios rítmicos en muchos procesos fisiológicos, mentales y conductuales en el organismo. En la actualidad son cada vez hay más las pruebas que vinculan la cronodisrupción (alteración de los ritmos circadianos) con una funcionalidad aberrante en la expresión de los genes del reloj, lo que da lugar a múltiples enfermedades entre las que se incluye el cáncer.

El carcinoma de células escamosas de cabeza y cuello (HNSCC) es uno de los cánceres más comunes a nivel global. Además, a pesar de los avances en las técnicas de diagnóstico y en los tratamientos, la tasa de supervivencia continúa siendo del 60%, debido ello a la resistencia a fármacos y a la recurrencia. Por esta razón, se evidencia la necesidad en la búsqueda de nuevas dianas terapéuticas y fármacos frente a este tipo de tumores.

Una posible diana terapéutica son las mitocondrias debido a su papel crítico para el crecimiento tumoral y la metástasis. Su biogénesis y actividad se encuentra estrechamente regulada por los ritmos circadianos mediante el control de los genes reloj. Incluso, en los últimos años se ha demostrado que la fosforilación oxidativa regula la expresión de genes reloj clave, por lo que se establece un complejo circuito de retroalimentación entre la bioenergética celular y el mecanismo de relojería molecular.

La melatonina, cuya producción y secreción oscila según el ciclo de luz-oscuridad, es el principal regulador de la maquinaria circadiana. Además, los efectos oncostáticos de la melatonina se correlacionan con un aumento de la actividad mitocondrial. Sin embargo, los vínculos directos entre la expresión del gen del reloj circadiano, la actividad mitocondrial y los efectos antiproliferativos de la melatonina en los cánceres, incluido el HNSCC, siguen siendo en gran medida desconocidos.

Por esta razón, el objetivo de este estudio fue analizar la conexión entre la desincronización de genes del reloj y la disfunción mitocondrial en células cancerosas escamosas de cabeza y cuello, y analizar los efectos de la melatonina en estas células.

Para ello analizamos los efectos de la melatonina en líneas celulares HNSCC (Cal-27 y SCC9), que fueron tratadas con melatonina 500 y 1000 µM, en la expresión de los genes reloj *Bmal1* y *Per2*, así como en *Sirt1*, regulado de forma circadiana, el cual juega un papel clave en el metabolismo mitocondrial. Además, estudiamos el efecto de esta hormona en el consumo de oxígeno mitocondrial mediante electro de Clark.

En este trabajo descubrimos que la melatonina a altas dosis regula, de forma circadiana la expresión de los genes *Per2* y *Sirt1*. A su vez, demostramos que los efectos antiproliferativos de la melatonina no están mediados por el gen reloj *Bmal1*. Sorprendentemente, el efecto resincronizador de la melatonina sobre *Per2* y Sirt1 no produjo alteraciones en la oscilación de la actividad respiratoria mitocondrial. Por lo tanto, estos resultados permiten aumentar nuestra comprensión de los oncostáticos en el cáncer de cabeza y cuello, poniendo el foco en los efectos de esta hormona sobre la maquinaria circadiana. Además, esta investigación permite expandir los conocimientos actuales de los posibles mecanismos antiproliferativos de la melatonina en el tratamiento del HNSCC sugiriendo que sus efectos antiproliferativos son independientes de uno de los principales genes reloj, *Bmal1*.

SUMMARY

Circadian rhythms are a regulatory system, with an approximate periodicity of 24 hours, that generates rhythmic changes in many physiological, mental and behavioral processes in the body. There is now increasing evidence linking chronodisruption (alteration of circadian rhythms) with aberrant functionality in the expression of clock genes, which gives rise to multiple diseases, including cancer.

Head and neck squamous cell carcinoma (HNSCC) is one of the most common cancers globally. Furthermore, despite advances in diagnostic techniques and treatments, the survival rate continues to be 60%, due to drug resistance and recurrence. For this reason, the need is evident in the search for new therapeutic targets and drugs against this type of tumors.

A possible therapeutic target is mitochondria due to their critical role for tumor growth and metastasis. Its biogenesis and activity is closely regulated by circadian rhythms through the control of clock genes. In recent years, it has even been shown that oxidative phosphorylation regulates the expression of key clock genes, establishing a complex feedback loop between cellular bioenergetics and the molecular clockwork mechanism.

Melatonin, whose production and secretion oscillate according to the light-dark cycle, is the main regulator of the circadian machinery. Furthermore, the oncostatic effects of melatonin are correlated with an increase in mitochondrial activity. However, direct links between circadian clock gene expression, mitochondrial activity, and the antiproliferative effects of melatonin in cancers, including HNSCC, remain largely unknown.

For this reason, the objective of this study was to analyze the connection between clock gene desynchronization and mitochondrial dysfunction in head and neck squamous cancer cells, and to analyze the effects of melatonin on these cells.

To do this, we analyzed the effects of melatonin in HNSCC cell lines (Cal-27 and SCC9), which were treated with 500 and 1000 µM melatonin, on the clock genes *Bmal1* and *Per2*, as well as on the circadian-regulated *Sirt1*, which plays a key role in mitochondrial metabolism. In addition, we studied mitochondrial oxygen consumption using Clark electrode.

In this work, we discovered that melatonin at high doses regulates the expression of the *Per2* and *Sirt1* genes in a circadian manner. In turn, we demonstrate that the antiproliferative effects of melatonin are not mediated by the clock gene *Bmal1*. Surprisingly, the resynchronizing effect of melatonin on *Per2* and *Sirt1* did not produce alterations in the oscillation of mitochondrial respiratory activity. Therefore, these results allow us to increase our understanding of oncostatics in head and neck cancer, focusing on the effects of this hormone on the circadian machinery. Furthermore, this research allows us to expand the current knowledge of the possible antiproliferative mechanisms of melatonin in the treatment of HNSCC, suggesting that its antiproliferative effects are independent of one of the main clock genes, *Bmal1*.

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INTRODUCTION

1. CYRCADIAN RHYTHMS AND CLOCK GENES

Circadian rhythms are a fascinating and intricate aspect of our biology that govern the timing of various physiological and behavioral processes within our bodies. The concept of circadian rhythms was first elucidated by Halberg F. in 1959 (Halberg, 1959) to detail the connection between the Earth's daily rotation and the inherent oscillations of numerous physiological factors. Derived from the Latin words "circa," meaning around, and "diem," meaning day, circadian rhythms are essentially our body's internal clock that operates on a roughly 24-hour cycle. These rhythms are not exclusive to humans; they are found in most living organisms, from plants to animals, and play a crucial role in regulating various bodily functions. In mammals, the circadian clock acts as a regulatory system modulated by endogenous factors such as genetics or endocrine secretions, behavioral factors such as feeding/fasting, as well as external oscillating signals such as light/darkness and temperature cycles (Serin & Acar Tek, 2019). All these inputs generate rhythmic changes in many physiological processes, including the endocrine system, the cell cycle, DNA damage repair, metabolism, and the sleep/wake cycle.

The circadian clock is controlled by a central circadian pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus, which, in turn, acts hierarchically to synchronize all the peripheral oscillators, located in practically all cells in the body. The light:dark cycle, or photoperiod, is the main *Zeitgeber* (from the German time giver) for circadian entrainment in most animals, which gives rise to a regulation of peripheral clocks. In vertebrates, circadian photoreception is since the retina possesses specialized photosensitive retinal ganglion cells (ipRGCs) using melanopsin as a visual pigment, that is directly active by blue light with a wavelength of 440 to 480 nm (Wirz-Justice et al.,

2021). Subsequently, this signal is projected through the retinohypothalamic tract to the SCN. The SCN, in mammals, projects to a wide range of brain areas involved in the regulation of metabolic pathways; among them are the subparaventricular zone, dorsomedial hypothalamus, arcuate nucleus and the paraventricular nucleus (PVN) of the hypothalamus, allowing, the latter, its connection with the pineal gland (PG). In turn, the SCN sends two main efferent signals, homeostatic and chronobiotic ones (Buijs et al., 2021). The first one, project mainly to the hypothalamus targeting the autonomic and neuroendocrine systems, while the second one constitutes the main signal to control the pineal synthesis of melatonin, which in turn feedback on clock genes. Specifically, during the night, the SCN neurally signals the pineal gland promotes melatonin secretion, that peaks between 2 am and 4 am, and is rapidly released into de blood and cerebrospinal fluis reaching all cells of the body. Light increases the electrical activity of the SCN through photostimulation of ipRGC, producing the release of gamma-aminobutyric acid (GABA) in the PVN and, therefore, inhibiting melatonin secretion during the day (Kalsbeek et al., 2000; Wirz-Justice et al., 2021). Due to these properties, melatonin functions as an endogenous synchronizer of circadian rhythms since it transmits timekeeping signals from the SCN and coordinates each cell in the organism with a period of 24 hours. The synchronization of the cells in different tissues requires the precise coordination of the circadian clock. However, although circadian rhythms are fundamental to the correct physiological functioning of the organism, these mechanisms can be lost or weakened due to different factors, such as aging or various pathologies (Manoogian & Panda, 2017).

At the molecular level, the central circadian clock is composed of a small number of genes whose expression forms a time-delayed transcription–translation feedback

loop (TTFL) (Figure 1). The activating arm of the loop is composed of circadian locomotor output cycle kaput (CLOCK)/neuronal PAS domain protein 2 (NPAS2) and brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1), which form a heterodimer and connect with its promoter at CACGTG E-box sequences. The binding to the E-box by this heterodimer induces clock-controlled gene (CCG) expression. This facilitates the transcription of numerous genes, including the Cryptochrome (*cry1* and *cry2*) and Period (*per1*, *per2* and *per3*) genes. The CRY and PER proteins form a repressor complex that is translocated into the nucleus during the evening and physically interacts with the CLOCK/NPAS2:BMAL1 heterodimer in order to repress their own transcription. Subsequently, these proteins, which increase and accumulate in the cytoplasm, are phosphorylated by cysteine kinase 1 ε/δ (CK1 ε/δ) during the night for its degradation. They are targeted for ubiquitination by specific E3 ligases and are eventually degraded by the proteasome, thus further increasing their synthesis at the beginning of the day. The waxing and waning of this transcriptional feedback loop, which takes ~24 hours to complete, represents the core mechanism of the circadian clock in mammals (Partch et al., 2014; Patke et al., 2020).

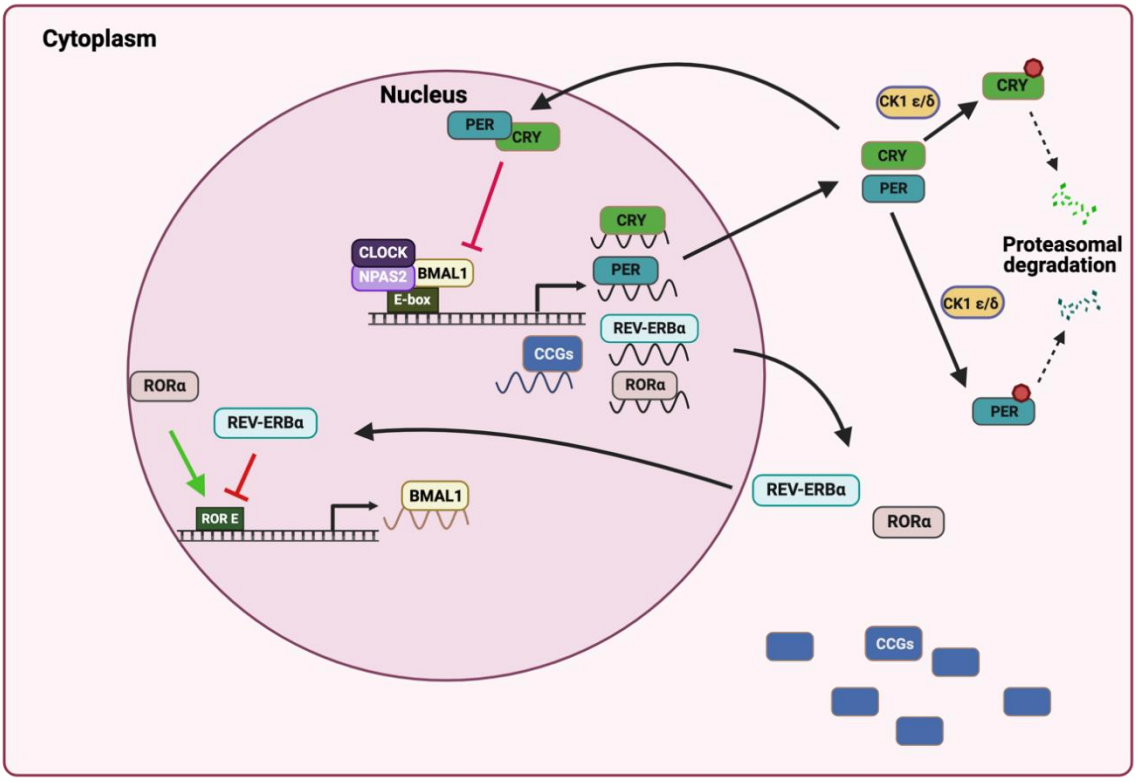

Figure 1. Schematic model of mammalian circadian clock mechanism. The molecular clock is composed of interconnected transcription feedback loops: the transcription factors CLOCK/NPAS2 and BMAL1 produce a heterodimer that binds to the E-box in the promoter and activates the transcription of Per, Cry, Rev-erbα, Rorα and CCGs. CRY and PER dimerize and enter the nucleus, where CLOCK-BMAL1-activated transcription is inhibited, thus generating an oscillatory pattern of gene expression. In the cytoplasm, PER and CRY are phosphorylated by CK1 ε/δ for its degradation. The REV-ERBα receptor inhibits Bmal1 expression, while RORα positively regulates Bmal1 expression. Brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (Bmal1); cysteine kinase 1 ε/δ (CK1 ε/δ); circadian locomotor output cycles kaput (Clock); cryptochrome (Cry); neuronal PAS domain protein 2 (NPAS2); Period (Per); reverse strand of protein ERB alpha (Rev-erbα); orphan retinoic acid receptor-related alpha (RORα) (Rodríguez-Santana et al., 2023).

In recent years, CHRONO (chip-derived repressor of network oscillator) has been identified as a key player in CLOCK–BMAL1 regulation. As with the CRY proteins, CHRONO inhibits CLOCK–BMAL1 on E-boxes, but with a distinct epigenetic mechanism, in a histone deacetylase-dependent manner (Hatanaka & Takumi, 2017).

1.1 CYRCADIAN RHYTHMS IN HEALTH

As a result of body clocks, organisms adjust their behavior and physiology based on recurring environmental changes associated with day and nighttime (for example, food availability, temperature, predator presence, etc.). Consequently, circadian rhythms can be found in virtually every aspect of behavior (e.g., sleep/wake cycle, fasting/feeding cycle), physiology (e.g., hormone secretion, immune response), and metabolism (e.g., glycolysis, fat metabolism) (Hut & Beersma, 2011; Meléndez-Fernández et al., 2023). However, as a result of our lifestyles, habits that cause deregulation of circadian rhythms (chronodisruption) are more common. Then, chronodisruption refers to the misalignment of internal clocks with *zeitgebers*. This phenomenon is common in people who experience jet lag, use artificial light at night, or have night jobs. In fact, according to worldwide epidemiological data, up to 30% of the working population are employed in non-standard work hours (for example, evening or rotating shifts) (Parent-Thirion et al., 2007). This causes significant alterations in sleep and biological functions which, in turn, can affect the physical and psychological well-being of people. There is accumulating evidence that living in a mismatch between your inner clock time and the external daytime time in the long-term provokes a wide range of pathologies including, metabolic and cardiac diseases, cognitive impairment, immune dysfunction, and an increased risk of cancer, among others (Buijs et al., 2021; Meléndez-Fernández et al., 2023; World Health Organization, 2010).

1.1.1 CHRONODISRUPTION AND CANCER

Alterations in clock genes are associated with different types of human cancer (Gu et al., 2017; Rahman et al., 2019; Relles et al., 2013; Tokunaga et al., 2008). However,

it is not understood how circadian disruption is associated with serious adverse health outcomes, including carcinogenesis.

The World Health Organization currently recognizes night shift work, as a probable carcinogen, classified as type 2A by the Agency for Research on Cancer (IARC) (World Health Organization, 2010). Since the 1980s, numerous epidemiological studies have linked an increased risk of different cancers, such as breast and prostate cancer, with night shift work by employees such as nurses and flight attendants on transatlantic flights (Cadenas et al., 2014; Conlon et al., 2007; Fagundo-Rivera et al., 2020; Touitou et al., 2017). It has been suggested that sleep deprivation, the light-induced suppression of melatonin and lifestyle changes are important mechanisms that could explain the possible link between shift work and cancer risk (World Health Organization, 2010). Moreover, studies of genetic variations in the circadian pathway in human clock genes have reported that the disruption of circadian rhythms is related to an increased risk of cancer (Mocellin et al., 2018). In support of these findings, a significant relationship has been established between different types of cancer, such as breast, lung and prostate cancer, and variations in *Bmal1*, *Clock*, *RORα* and *RORβ*. This is due to the fact that clock genes regulate between approximately 50% and more than 80% of mammalian genome genes, including the tumor suppressor gene *p53* and the oncogene *c-Myc* (Dang, 2012; Gotoh et al., 2015). The disruption in the circadian clock is therefore involved in tumor development by altering the expression of genes involved in fundamental functions, such as the cell cycle, apoptosis, metabolism and energy, DNA repair, tumor immunity and metastasis (Li, H.X 2019). In addition, the polymorphisms of other clock genes specific to each tumor type have been identified, suggesting that certain circadian genes

might be more important than others in terms of predisposition to different types of cancer (Mocellin et al., 2018).

Intriguingly, given that cancer prognosis and survival have been associated with the level of circadian disruption in patient tumor tissues (Kiessling et al., 2017), clock genes, such as *Bmal1*, *Per1* and *npas2*, could be considered potential prognostic biomarkers in certain cancers (Mazzoccoli et al., 2011; Yi et al., 2010).

Nevertheless, other studies found no evidence of a relationship between interruptions in the circadian rhythm in night shift workers and carcinogenesis (Barul et al., 2019). Recently, in a meta-analysis of 57 observational studies, no evidence of a relationship was reported between night shift work and an increased risk of cancer, especially in breast, prostate, ovarian, pancreatic, colorectal, non-Hodgkin's lymph and stomach cancers (Dun et al., 2020). However, these differences in results can be explained by the methodological differences of the different epidemiological studies, as well as in the statistical tools used (Erren & Lewis, 2017). Therefore, common criteria to clarify the relationship between an alteration in circadian rhythms and carcinogenesis are clearly required.

1.1.1.1 CLOCK GENES, PROLIFERATION AND APOPTOSIS

In the intricate world of biology, cell proliferation and apoptosis are two fundamental processes that determine the destiny of every cell in our bodies. These processes are essential for growth, development, tissue repair, and maintaining the delicate balance required for health and homeostasis. These intricate mechanisms involve precise regulation which ensures that the body's tissues and organs can function optimally. However, the regulation of both mechanisms is complex and strictly

controlled which, when deregulated, can have serious health consequences. Malignant tumors are characterized by uncontrolled cell proliferation, partly due to a loss of control of cell cycle events caused by clock gene dysregulation (Soták et al., 2014). Clock genes affect many biological pathways, including those involved in cell proliferation and apoptosis, by controlling the expression of cell cycle genes (H. X. Li, 2019). In addition, substantial evidence shows that progression through the cell cycle occurs at specific times of the day/night cycle, suggesting that one function of the circadian clock system is to control this fundamental process (Gréchez-Cassiau et al., 2008). It is remarkable that circadian rhythms share some common features with the cell cycle, while disruptions in circadian clocks have been found to be related to carcinogenesis. For example, the BMAL1-CLOCK/NPAS2 heterodimer has been reported to repress c-Myc, an oncogene that contributes to the genesis of many human cancers, and whose protein expression is closely correlated with cell proliferation rates (Bretones et al., 2015; Kiessling et al., 2017). REV-ERBα and RORα have also been reported to regulate the expression of *p21*, a CCG that negatively regulates cell cycle progression (Gréchez-Cassiau et al., 2008). Overexpressed PER1 also induces *c-Myc* and suppresses *p21*, while other studies have reported that PER1 inhibits *Cyclin B1*, *Cdc2* and *Wee1* expression, leading to a decrease in cancer cell proliferation (Gery et al., 2006). This discrepancy may be due to differences in the methods used or in the characteristics of the cell lines used in the experiments (Sato et al., 2011). Nevertheless, these data indicate that many genes crucial to the cell cycle are under the control of clock genes that are aberrantly expressed in many tumor tissues.

It has also been reported that many crucial genes involved in cell proliferation and apoptosis have periodic patterns of expression. These genes, which oscillate during

a 24-h cycle, include the proliferation gene *Ki-67*, the tumor suppressor gene *p53*, the proto-oncogene Mdm2 and the apoptotic-related proteins BAX and BCL-2 (H.-X. Li et al., 2016). PER1 and PER2 have been reported to be mainly associated with the upregulation of *Ki-67*, *Mdm2* and *Bax*, as well as with the downregulation of *Bcl-2*, c-Myc and *p53*, in lung, pancreatic, hepatocellular, and oral carcinoma cell lines (Hua et al., 2006; H.-X. Li et al., 2016; Sato et al., 2009). In addition, there is a strong connection between Bmal1 and Per2 regulation and the PI3K/mTOR signaling pathway, one of the most frequently activated signaling pathways in tumorigenesis and the progression of cancer. For example, BMAL1 depletion leads to cell cycle disruption, which results in a substantial increase in the apoptotic cell population, and to the acceleration of cell invasion Matsumoto, C.S 2016). The PI3K/mTOR pathway has a major effect on the regulation of processes such as autophagy, proliferation, and apoptosis, which sequentially affects the occurrence and development of cancer (H. Liu et al., 2020; Matsumoto et al., 2016; Z. Wang et al., 2020).

Many signaling pathways cooperate with clock genes involved in tumorigenesis; the altered expression of these genes can modify a range of downstream CCGs and tumor-related genes, which impacts tumor cell proliferation, apoptosis, migration, and invasion (Sancar & Van Gelder, 2021).

Collectively, these data suggest that the circadian clock can control cell proliferation and apoptosis at multiple levels and that disruption of the circadian system is linked to tumor cell growth.

1.1.1.2 CLOCK GENES AND METASTASIS

Metastasis, the process by which cancer cells spread from their primary site to distant organs, represents a critical phase in cancer progression. This intricate and multi-step journey is characterized by the escape of malignant cells, their circulation through the bloodstream or lymphatic system, and the establishment of secondary tumors. The relationship between metastatic disease progression and circadian rhythms has been poorly characterized (Diamantopoulou et al., 2022; Gwon et al., 2020; J. Wang et al., 2019). However, a large number of studies have demonstrated the participation of clock genes in metastasis. For example, low PER1/PER2 expression in different types of cancer, such as breast (Broadberry et al., 2018), glioma (Xia et al., 2010), gastric (Zhao et al., 2014) and non-small cell lung cancer (Xiang et al., 2018), is closely related to the development and metastasis of tumors. Moreover, much evidence has identified BMAL1 as a key element in metastasis in breast cancer and glioblastoma. BMAL1 regulates the expression and activity of matrix metalloproteinase 9 (MMP9), which controls cell migration and invasion (Gwon et al., 2020; J. Wang et al., 2019). MMP9, which is involved in the degradation of the tumor extracellular matrix, is a mediating factor regarding the local invasion and distant metastasis of tumor cells. In human colorectal cancer, BMAL1 has been shown to induce metastasis by stimulating exosome secretion (Dong et al., 2022). Exosomes derived from primary tumors have been shown to alter the microenvironment of secondary organs in order to facilitate the colonization and growth of metastatic tumors, whose quantity is dependent on circadian rhythms (Dong et al., 2022).

1.1.1.3 CLOCK GENES AND TUMOR IMMUNITY

The relationship between the immune system and cancer has been a subject of extensive research in recent years. Tumor immunity, the intricate interplay between the immune system and cancer cells, plays a pivotal role in shaping the course of cancer development and response to therapy. Circadian rhythms are also involved in the mammalian immune system, which involves various populations of immune cells, such as monocytes, natural killer cells, dendritic cells (DCs) and T and B lymphocytes, as well as responses to signals and their defensive functions, including cytokine levels (Scheiermann et al., 2018; C. Wang et al., 2023). Thus, when circadian homeostasis is disrupted, deregulation of the immune system produces immune suppression and the accelerated development of tumors. BMAL1 is the principal mediator of the circadian control of the immune system and also promotes anti-inflammatory states. Downregulation of BMAL1 has been found in hematologic malignancies such as diffuse large B-cell lymphoma, as well as acute lymphocytic and myeloid leukemias (Taniguchi et al., 2009). Deletion of BMAL1 affects the development of B lymphocytes (Sun et al., 2006). In addition, a recent investigation by Wang, C. et al. (C. Wang et al., 2023) showed that, in murine models with the specific inhibition of *Bmal1* in T and DCs, regardless of the time at which melanoma cells were inoculated, the tumor volume was similar after 14 days of the experiment. However, in wild-type mice, significant differences in tumor growth were observed depending on the time of engraftment. Therefore, BMAL1 and cell-autonomous circadian oscillations in both DCs and T cells are critical for time-of-day differences in tumor volume.

BMAL1, CLOCK, REV-ERBα and RORα have also been reported to regulate immune functions and inflammation by modulating CCGs that encode a variety of

proteins, including cytokines, chemokines, and receptors (H. X. Li, 2019). However, the central pacemaker is also modulated by immune factors such as proinflammatory cytokines and IL-1/6, as well as by anti-inflammatory drugs, at the molecular and cellular levels, which results in the subsequent alteration of clock genes. In conclusion, the circadian clock and immune system can be said to exert bidirectional control (Cermakian et al., 2013).

1.1.1.4 CHRONOTHERAPY IN CANCER

The toxicity, efficacy and even the pharmacokinetics or metabolism of a drug can vary with the time of day, depending on its mechanism of action (D. P. Cardinali et al., 2021). In recent years, special attention has been paid to the administration of anticancer drugs depending on circadian rhythmicity in order to maximize efficacy and to reduce side effects. For example, there is evidence that, in synchronized esophageal cancer cells, DNA damage induced by cisplatin is greater when coinciding with lower levels of PER2 (Redondo et al., 2021). In *in vivo* melanoma models, mice treated at night showed a higher rate of cisplatin–DNA adduct removal and less toxicity than those treated in the morning, which coincides with maximal global and gene-specific repairs. Interestingly, differences in the effects of this treatment were not observed in Per1/2 knockout mice (Dakup et al., 2018).

Chronotherapy has improved the implantation of medication for other pathologies, such as asthma (D. P. Cardinali et al., 2017; Thakur et al., 2019), osteoarthritis and rheumatoid arthritis (Whibley et al., 2019). However, to date, studies carried out to evaluate chronochemotherapy for some types of cancer are contradictory. For example, chronotherapy in the case of ovarian cancer has shown no

beneficial effect (Lévi et al., 1990), while male patients with colorectal cancer lived significantly longer (Giacchetti et al., 2012).

The main problem concerning the clinical application of chronotherapy is the need for studies of the circadian gene expression profiles of specific cancers. In addition, non-invasive methods to assess the circadian parameters of cancers need to be developed. Thus, although the use of chronotherapy in cancer treatment is an interesting avenue of study, more in-depth research is necessary (Sancar & Van Gelder, 2021).

Therefore, given all these data, the circadian clock clearly plays a fundamental role in tumor pathogenesis. Alterations in clock gene expression correlate with alterations in DNA replication, DNA repair and responses to DNA damage in the metabolism and in senescence. The control of cell proliferation and apoptosis is also lost, metastasis spreads, the immune system is altered and even drug resistance increases. Thus, the “repair” of circadian rhythms has been shown to be a possible therapeutic target against this disease. For this reason, in recent years interest in chronodisruption has increased in recent years.

1.2 CIRCADIAN RHYTHM AND MITOCHONDRIAL FUNTION

Energy metabolism is modulated by the circadian rhythm which precisely regulates the expression, secretion and/or activation of many hormones, enzymes, and transport systems. Numerous studies have established direct control of biological clock genes on mitochondrial function even in the absence of external signals or neuroendocrine signals. The main connection is found in BMAL1, which induces the expression of nicotinamide phosphoribosyltransferase (NMAPT), an enzyme that

synthesizes nicotinamide adenine dinucleotide (NAD+), a substrate for sirtuins (Imai, 2010). The sirtuins are a NAD+ dependent family of histone deacetylases (HDACs), which are implicated in various physiological functions ranging from aging, maintenance of genome integrity, stress response, metabolic control, and cancer (Q. J. Wu et al., 2022). Mammalian sirtuins play a relevant role in modulating the circadian epigenome and provide specificity in transcriptional control. Interestingly, the seven mammalian sirtuins (SIRT1-7) vary in their enzymatic activity (apart from their deacetylase function), biological targets, and cellular function. The most studied sirtuin is SIRT1, a nuclear protein involved in metabolism control. Because the metabolism and deacetylase activity of SIRT1 oscillates with a daily period, in 2008, Nakahata Y. et al. (Nakahata et al., 2008) demonstrated an association between BMAL1, CLOCK and SIRT1. It was proposed that SIRT1 interacts directly with CLOCK and interacts with the BMAL1:CLOCK complex, controlling circadian histone acetylation by CLOCK (a histone acetylase (HAT)). The circadian function of BMAL1 is regulated by its acetylation by CLOCK and deacetylation of BMAL1 by SIRT1 has been suggested to contribute to its activity. However, the activity of SIRT1 depends on the oscillating levels of NAD+, synthesized in the salvage pathway by the committing NAMPT, whose expression shows a circadian rhythm, which means that NAMPT, NAD+, and SIRT1 activity are under circadian control, constituting a loop with BMAL1:CLOCK complex. However, although today the circadian relationship of the rest of the sirtuins is not so evident, in recent years there is more and more evidence that supports it, as is the case of SIRT3 or SIRT6 (Peek et al., 2013).

Interestingly, SIRT1, can also directly interact and deacetylate peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). PGC-1 transcriptional coactivators are important regulators of several crucial aspects of energy

metabolism. PGC-1α controls many aspects of oxidative metabolism, including mitochondrial biogenesis and redox homeostasis. In this way, a complex feedback mechanism is established where through the clock genes, especially BMAL1, the acetylation and deacetylation of PGC-1α is allowed through SIRT1, regulating its activity (C. Liu et al., 2007). On the other hand, SIRT3, detected mainly in mitochondria, has a key role in cellular bionergetics by regulating the electron transport chain (ETC) or the production of acetyl-CoA. Furthermore, it has an important function in modulating mitochondrial oxidative stress by regulating superoxide dismutase 2 (SOD2), deacetylating and activating the transcription factor FOXO3a, as well as directly deacetylating and activating the activity of SOD2 dismutase (Florido, Rodriguez-Santana, et al., 2022). Therefore, consistently, recent studies have addressed the role that bioenergetics and mitochondrial dynamics play in cellular metabolism in relation to circadian rhythmicity.

Recently, Nazzareno Capitanio's research group (Cela et al., 2016; Scrima et al., 2016) have described a new intertwined transcriptional enzymatic feedback loop that controls the molecular interplay between cellular bioenergetics and molecular clockwork. In this way, they demonstrated the loop in which BMAL1 is indispensable in the circadian activity of oxidative phosphorylation (OxPhos) and how inhibition of OxPhos produces dramatic deregulation of rhythmic clock gene expression. Furthermore, they concluded that the respiratory activity itself presented a rhythmicity due to the reversible acetylation of a single subunit of Complex I of the mitochondrial respiratory chain due to the circadian action of SIRT1/3.

2. ROLE OF MITOCHONDRIA IN CANCER

Tumor metabolism has been the subject of numerous investigations as it is one of the main characteristics that differentiate normal cells from tumor cells. The alteration of metabolism in the tumor cell is related to the malignancy of these cells. In fact, tumor cells activate some metabolic pathways, while inactivating others, increasing their proliferative capacity. As a result of metabolic reprogramming, tumor cells can satisfy the high energy demand caused by cell proliferation and maintain redox balance within. Consequently, in recent years several studies have focused on tumor metabolism and even proposed using it as a therapeutic target against cancer (DeBerardinis & Chandel, 2016).

In 1956, Otto Warburg described, for the first time, the mechanism by which tumor cells obtain energy. Tumor cells, even in the presence of oxygen, mainly use lactic fermentation to obtain the energy necessary for their growth, instead of performing aerobic respiration (Warburg effect) (Warburg, 1956). That is, tumor cells obtain ATP from glucose by producing mainly lactate, instead of carrying out a complete oxidation of glucose through respiration in the mitochondria, producing CO2. Therefore, this characteristic makes tumor cells more susceptible to mitochondrial changes.

Mitochondria, often referred to as the cellular powerhouses, have long captivated the scientific community with their essential role in energy production by using the electron transport chain (ETC) through OxPhos. However, in recent years, our understanding of mitochondria has evolved significantly, revealing their involvement in a myriad of cellular processes beyond ATP generation. In addition to the role that mitochondria play in metabolic reprogramming, numerous studies have linked mitochondrial dysfunctions with multiple characteristics of cancer such as unlimited

proliferative potential, insensitivity to anti-growth signals, altered apoptosis, increased anabolism, and decreased autophagy. Mitochondria are also involved in the regulation of important processes such as cell death, inflammation, immunity, and migration. All of this could explain the importance of the role of mitochondria in the tumor process (Giampazolias & Tait, 2016).

2.1 MITOCHONDRIA AND CELL DEATH

Mitochondria play a central role in the intricate regulation of multiple fundamental processes in cell life and death, such as apoptosis, necrosis, necroptosis, ferroptosis, pyroptosis (Bock & Tait, 2020) or the activation of proinflammatory pathways (Qi et al., 2023; Vringer & Tait, 2023). Mainly in apoptosis, this organelle acts by focusing on the release of apoptogenic factors, the regulation of mitochondrial outer membrane permeabilization (MOMP) and the balance between pro-apoptotic and anti-apoptotic proteins. Apoptotic signaling involves the release of cytochrome c from mitochondria and subsequent caspase activation. However, MOMP permeabilization also involves the activation of proinflammatory pathways (Qi et al., 2023). Understanding this complex relationship is therefore essential to unraveling the secrets of cell fate and holds great promise for therapeutic interventions in conditions ranging from neurodegenerative disorders to cancer.

Tumor cells present an inhibition of mitochondrial apoptosis for various reasons. Firstly, tumor cells present an inhibition of the tumor suppressor p53, a nuclear transcript factor that stimulates apoptosis (Hassin & Oren, 2023). Suppression of p53 is mainly due to individual genetic alterations of the TP53 gene and are recognized as driving events in many types of human tumors. On the other hand, tumor cells also avoid

apoptosis by inhibiting the function of caspases. Although this is considered a point of no return, tumor cells can survive these conditions by inactivating caspases, keeping mitochondria intact (Lopez & Tait, 2015). Furthermore, tumor cells tend to block apoptosis by increasing the expression of anti-apoptotic Bcl-2 proteins. This is because the permeabilization of the outer mitochondrial membrane is a point of no return and is regulated by proteins of the Bcl 2 family. In this aspect, several subfamilies of proteins are distinguished: the subfamily of proapoptotic proteins such as Bax and Bak, as well as the BH3-only proteins, and the Bcl-2 antiapoptotic protein subfamily. The activation of Bax and Bak is essential for the permeabilization of the outer mitochondrial membrane to occur since, once active, these proteins oligomerize in the outer mitochondrial membrane, forming pores in it. On the other hand, antiapoptotic proteins of the Bcl-2 family prevent the permeabilization of the outer mitochondrial membrane by binding and inactivating the Bax and Bak proteins. In this way, the balance between pro- and anti-apopototic proteins is responsible for the regulation of apoptosis (Lopez & Tait, 2015). The overexpression of these proteins is a common characteristic in various types of cancers and is produced by a higher number of copies of the gene, transcriptional overexpression or deregulation of microRNAs that suppress the expression of these anti-apoptotic proteins. In addition, a lower expression of Bax or Bak proteins has also been observed.

On the other hand, in tumor cells, mitochondria play a key role in the inflammation process, a key process in tumorigenesis. It has been shown that approximately 25% of cancers are related to chronic inflammation due to many factors such as bacteria, viruses or non-living molecules such as asbestos and tobacco smoke. Although inflammation clearly has tumor inhibitory functions, inflammation has

oncogenic effects in many contexts resulting in unlimited replicative potential, evasion of apoptosis and resistance to growth inhibitory factors, and increased angiogenesis and metastasis, due to modifying effects. of the matrix (J. Hou et al., 2021; Khandia & Munjal, 2020; Murata, 2018). Briefly, mitochondrial components such as mtDNA or mitochondrial ROS present in the cytosol activate the NLRP3 inflammasome and the release of proinflammatory enzymes (Murata, 2018). All of this reduces immune surveillance against the tumor and even, when tumor cells have very damaged mtDNA, they can exchange mitochondria with adjacent immune cells and thus improve their survival. In short, the role of inflammation is complex but key in the development of new cancer treatments (Giampazolias & Tait, 2016).

2.2 RELATIONSHIP BETWEEN MITOCHONDRIA AND OXIDATIVE STRESS

Reactive oxygen species (ROS), a family of chemically reactive molecules, have long been recognized as double-edged swords in cellular biology. They play vital roles in cellular signaling, redox regulation, and immune response, but excessive ROS production can lead to oxidative stress and damage to biomolecules. The main source of free radicals in most cell types are the mitochondrial ROS (mtROS) produced by the respiratory chain during oxidative phosphorylation. More specifically, the respiratory chain is the main source of ROS, with complexes I (CI; NADH:ubiquinone oxidoreductase) and III (CIII; ubiquinol:cytochrome c oxidoreductase; cytochrome bc1 complex) generally considered as the main sources of ROS, while the contribution of intact complex II (CII; Succinate dehydrogenase (SDH)) appears to be insignificant (Brand, 2010; Dröse, 2013).

However, recent studies highlighted the important role of reverse electron transport (RET) that produces mtROS (Dröse, 2013; Onukwufor et al., 2019)

In cancer, ROS also have a dual effect, where at low concentrations they act as signaling molecules and contribute to metastasis, resistance to apoptosis and angiogenesis. On the other hand, high levels of ROS are toxic, inducing cell cycle arrest, apoptosis, and senescence. Therefore, achieving a disproportionate increase in ROS, thanks to treatments such as chemo or radiotherapy, causes cell death. That is, the protumorigenic or antitumorigenic role of ROS depends on the levels at which they are produced (Moloney & Cotter, 2018). The regulation, therefore, of the concentration of ROS in tumor cells depends on antioxidant activity, which makes them extremely vulnerable. Its concentration depends on the activity of antioxidant enzymes such as superoxide dismutase (SOD), responsible for transforming $O_2^{\bullet-}$ into H_2O_2. In addition, other enzymes act such as catalase, which reduces H_2O_2 to water and molecular oxygen, and glutathione peroxidase (GPx), which eliminates H_2O_2 (Florido, Rodriguez-Santana, et al., 2022; Moloney & Cotter, 2018). For this reason, it becomes evident that the balance between the expression of antioxidant enzymes and the production of ROS is critical for tumor progression to occur since the tumor cell needs not too high ROS values for its survival.

To maintain the redox state and avoid cell death, tumor cells alter their metabolic pathways to generate antioxidant molecules (NADPH and reduced glutathione (GSH)) and cofactors (NADH and FADH2). To achieve this, tumor cells follow numerous strategies such as increasing glycolytic activity, increasing the pentose phosphate pathway, decreasing mitochondrial metabolism to avoid the production of ROS or activating nuclear factor erythroid 2-related factor 2 (Nrf2), which activates enzymes

that participate in the synthesis of GSH. They even increase glutaminolysis and the oxidation of fatty acids, allowing the production of NADPH.

3. HEAD AND NECK CANCERS

Head and neck cancer (HNC) encompasses a group of neoplasms or malignant tumors of the oral cavity, pharynx, larynx, paranasal sinuses, nasal cavity, and salivary glands (Figure 2). According to the World Health Organization (WHO), head and neck cancer is one of the cancers with the highest incidence worldwide. It is estimated that more than 550,000 new cases are diagnosed each year (Johnson et al., 2020; Kusampudi & Konduru, 2021; Pisani et al., 2020). Although a patient's prognosis depends on multiple factors, it is estimated that the survival rate is only around 60% at 5 years. The seriousness of this type of cancer lies in the fact that 2 out of 3 cases are detected in already advanced stages of the disease, since in the first phase the symptoms usually go unnoticed, causing a delay in diagnosis (*Spanish Group of Cancer Patients (GEPAC)*, 2023). In addition, these types of tumors are characterized by local recurrences, distant metastases and second primary tumors.

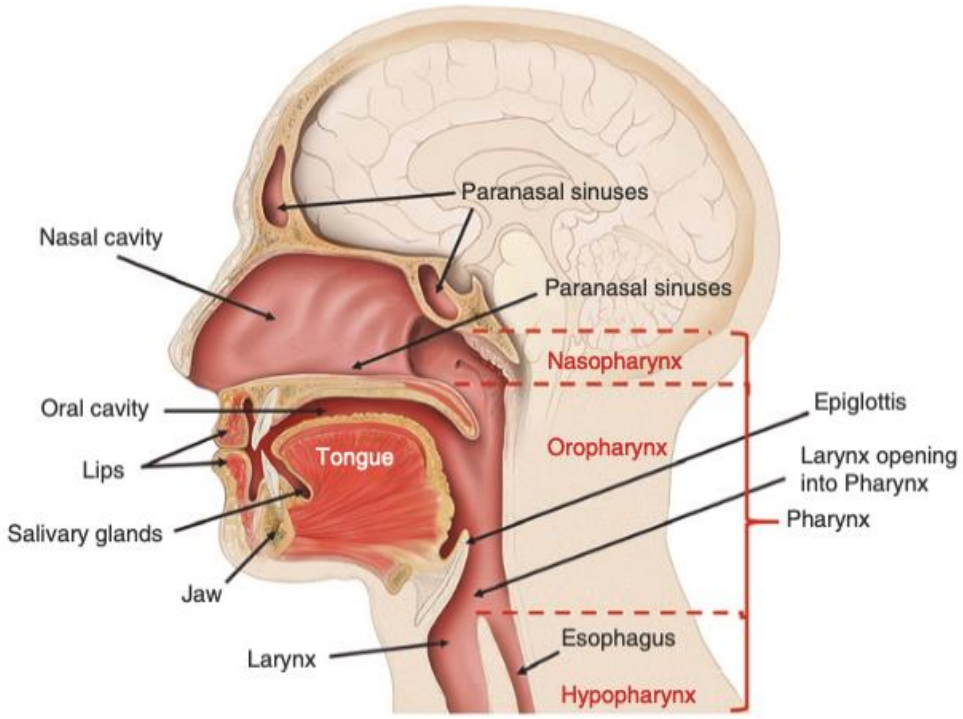

Figure 2. Regions of head and neck cancer (Kusampudi & Konduru, 2021).

Principally, head and neck cancers are squamous cell carcinomas (HNSCC) that arise in the epithelial lining of the cavities mentioned above, accounting for about 90% of all HNC (Kusampudi & Konduru, 2021). As a major risk factor for HNSCC, tobacco and alcohol consumption are the main causes, as well as various viral infections, particularly human papillomavirus 16 (HPV16), and genetic polymorphisms or damage. Generally, HNSCC is treated with chemotherapy, surgery for operable cases, and radiation therapy. Surgery with or without radiation therapy is considered as the primary treatment option whereas chemotherapy is usually prescribed as an adjuvant or supplementary treatment. Even though early-diagnosed cases tend to have a higher survival rate, advanced cases and those with metastases maintain an unchanged mortality rate. For that reason, despite advances in cancer research, it is necessary to find new therapeutic targets, develop new treatments and/or enhance and improve existing therapies.

Nevertheless, considering the fact that the molecular basis of HNSCC is rather complex and the intra-tumor heterogeneity is extraordinarily high, targeted therapy will probably yield limited solutions. Interestingly, the circadian system impairment has been identified in diverse types of cancers including breast, colorectal and prostate cancer. Therefore, a more systemic approach towards understanding the pathogenesis of HNSCC has recently been recognized and focuses on abnormalities in the functioning of the circadian system in humans.

3.1 NECK SQUAMOUS CELL CARCINOMA AT MOLECULAR LEVEL

Regarding cancer, this originates from genetic and epigenetic alterations in the genes that encode proteins that act on a variety of signaling pathways, giving rise to the different phenotypes associated with cancer. These genetic changes can be broadly defined as mutations, including alterations spanning gains and losses, sometimes of entire chromosomes or chromosome arms involving thousands of genes, down to genetic changes at the base pair level. The importance of these large gains and losses in cancer evolution remains somewhat difficult to understand, however, inactivating mutations in tumor suppressor genes appear to be of great relevance, indicating that they play a critical role. For thirty years, molecular data on cancer has increased rapidly, allowing us to better understand this complex disease (Leemans et al., 2018).

As with other cancers, the pathogenesis of head and neck cancers, including HNSCC, is a complex process that includes genetic mutations along with altered protein expression change the cellular microenvironment, leading to uncontrolled cell growth and the development of tumors. Furthermore, oropharyngeal tumors are differentiated

based on the risk factors that produced them. Thus, HPV-induced tumors are considered a separate pathological entity, which has recently been manifested in an adapted prognostic staging system. In 2015, The Cancer Genome Atlas (TCGA) consortium published a comprehensive molecular catalogue on HNSCC (Lawrence et al., 2015) allowing tumors to be subclassified according to their genomic profile. It shows that human-papillomavirus-associated tumours are dominated by helical domain mutations of the oncogene *PIK3CA*, allowing uncontrolled cell proliferation and growth via the PI3K pathway (Falasca, 2011; Plo, 2011). In addition, they are related to alterations that involve the loss of *TRAF3*, and amplification of the cell cycle gene *E2F1*. Smoking-related HNSCC, in contrast, demonstrate loss-of-function mutations in the tumor suppressor genes *p53* and *CDKN2A* (cyclin-dependent kinase inhibitor 2A). Furthermore, in tumor subgroups with favorable clinical outcomes, copy number alterations occur infrequently in combination with activating mutations of *HRAS* (gene encoding the p21 protein) or *PIK3CA* and inactivating mutations of *CASP8*, *NOTCH1* and *p53*. Besides mutations, genes can also be activated or inactivated by amplification (e.g., epidermal growth factor receptor (EGFR) amplification) or heterozygous and homozygous losses (e.g., CDKN2A losses) or epigenetic changes. However, it is currently difficult to understand the magnitude of molecular data available because of the overwhelming volume.

3.1.1 ASSOCIATION BETWEEN HEAD AND NECK SQUAMOUS CELL CARCINOMA AND CIRCADIAN CLOCK GENES

As mentioned earlier, circadian clock genes affect tumor development and prognosis by regulating downstream CCGs that are involved in cancer-related pathways. Several studies have reported the role of clock genes in head and neck carcinogenesis.

The first to provide evidence linking HNSCC development with alterations in circadian clock gene expression were Hsu et al. (Hsu et al., 2012a). In this study, Hsu et al. investigated the expression of nine circadian clock genes (*Bmal1, Clock, Per1, Per2, Per3, Cry1, Cry2, Tim, CKIε*) in adjacent tumor and non-tumor tissue from 40 patients diagnosed with HNSCC. This study allowed them to conclude several points of great interest, such as that the transcription levels of *Per*, *Cry*, *Bmal1* and *CKIε* were significantly decreased in cancerous tissues. In addition, these authors also found downregulated *Per3*, *Cry2*, and *Bmal1* expression was correlated with more advanced cancer stages. Subsequently, studying the peripheral blood of 94 patients they found a downregulation in *Per1* and *Clock*, suggesting these two proteins as possible circulating prognostic markers for HNSCC (Hsu et al., 2014).

Other studies have also found that variations in the circadian patterns of clock genes *Per1* and *Per2*, as well as tumor-related genes CyclinD1, CDK1, CyclinB1, p53, *c-Myc* and *VEGF* (vascular endothelial growth factor), have been associated with cancer development, suggesting that carcinogenesis can alter circadian patterns at the molecular and cellular level (X. M. Tan et al., 2015; Ye et al., 2015). It has even been reported that Per1 and Per3 have antiapoptotic effects and proapoptotic effects respectively in human gingival cancer (CA9-22) cells, through the Bim protein (Sato et al., 2011). Therefore, Per1 appears to play an important role in carcinogenesis because a gradual decrease has been observed as tumor development progresses in terms of clinical staging and lymph node metastasis (R. Chen et al., 2012).

Additionally, attention has been drawn to the clock gene *Per2*. In 2017, Xiong H and colleagues (Xiong et al., 2018) published a study where they studied oral squamous cell cancer (OSCC) tissues from 8 patients and paraffin-embedded tissue sections from

40 OSCC patients. In this study, the mRNA and protein expression levels of PER2 was significantly reduced, and the downregulation of PER2 accelerated the occurrence of OSCC metastasis and shortened the survival time of patients. PER2 expression was negatively correlated with PIK3CA and P53 levels, and positively correlated with PTEN, P14ARF and caspase-8 levels. Based on this, they conclude that PER2 could play an antitumor function through the P53/P14ARF, PIK3CA/AKT, and caspase-8 pathways. Moreover, studies where *Per2* is overexpressed in nasopharyngeal carcinomas demonstrate a decrease in proliferation, migration and invasion and improved apoptosis *in vitro*, demonstrating the relevance of this clock gene in the development of this disease (L. Hou et al., 2020).

It has even been reported that the clock gene *Bmal1* plays a key role in the efficacy of drugs such as paclitaxel in HSCC. Tang Q et al. demonstrated that increased Bmal1 inhibits cell proliferation, migration, and invasion in vitro and tumor growth in mouse xenograft models of HSCC. They further reported an increase in the population of apoptotic Bmal1-overexpressing cells after exposure of the cells to paclitaxel and improved paclitaxel sensitivity *in vivo*. Therefore, *Bmal1* is suggested as a tumor suppressor gene capable of increasing the sensitivity of cancer cells to paclitaxel and implies the possibility of taking Bmal1 into account as a biomarker of chronotherapy time.

3.1.2 ASSOCIATION BETWEEN HEAD AND NECK SQUAMOUS CELL CARCINOMA AND MITOCHONDRIAL METABOLISM

The relationship between mitochondria and cancer is a complex interaction that extends beyond the traditional view of mitochondria as mere energy producers. As we saw previously, mitochondria play a key role in tumor development, and the case of

head and neck squamous cell carcinoma is no exception. In recent years, some research has focused on understanding the mitochondrial metabolism of HNSCC for the development of new therapies against it. Recent meta-analyses of patients have revealed important implications of genes related to lipid and energy metabolism as predictors of survival (Bai et al., 2022; de Mattos et al., 2021; Hu et al., 2019). Although these studies did not reveal the direct role of mitochondria in these processes, considering the importance of mitochondria in metabolism, they undoubtedly also play a multitude of roles in HNSCC.

Furthermore, there is much evidence of the role of mitochondria in the development of HNSCC, such as the oxidation of NADH, a lactate derivative produced by cancer-associated fibroblasts (CAFs), in the OxPhos of OSCC cells could promote proliferation. Even the M2 isoform of pyruvate kinase (PKM2), a glycolytic enzyme that catalyzes the last step in glycolysis, the conversion of pyruvate and ATP from phosphoenolpyruvate and adenosine diphosphate (ADP), was strongly correlated with OSCC tumor progression (Kurihara-Shimomura et al., 2018). The important relationship of mitochondria with HNSCC implies that it is an important therapeutic target against this type of tumors and the problem of chemoresistance. In fact, a large part of the treatments tested against HNSCC focus on the mitochondria with the aim of improving the life expectancy of patients.

4. MELATONIN

Melatonin was first isolated and structurally identified chemically in bovine pineal tissue by Lerner et al. in 1958 (Lerner et al., 1958). This hormone is a highly conserved molecule found widely throughout nature. The origin of melatonin can be

traced back an estimated 2.5 billion years. Therefore, melatonin is a very old molecule, widely dispersed throughout different taxa. It is present in bacteria, unicellular eukaryotic organisms, algae, plants, invertebrate fungi and vertebrates, which suggests an important ancestral relationship of this indolamine with the physiology and biochemistry of cells.

Melatonin is a small molecule (Figure 3), derivative of the amino acid tryptophan, that display high lipid solubility which facilitates passage across cell membranes. Initially, pineal melatonin was characterized as a regulatory element in circadian rhythms. However, melatonin is also produced in other organs and tissues at higher concentrations (micromolar range) than that produced in the pineal gland (picomolar/nanomolar range), known as extrapineal melatonin (Acuña-Castroviejo et al., 2014).

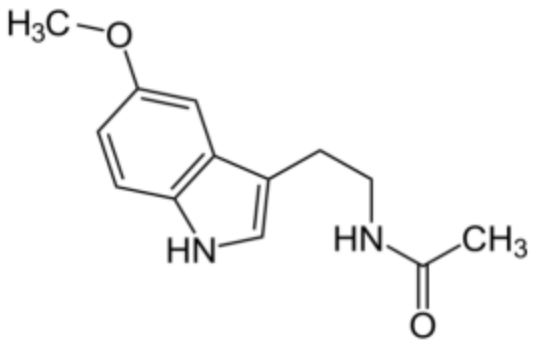

Figure 3. Schematic structure of melatonin.

4.1 MELATONIN SYNTHESIS

In 1960, Axelrod's group (Axelrod & Weissbach, 1960; Weissbach et al., 1960) reported the biosynthesis of melatonin for first time. Melatonin is synthesized from tryptophan and this process occurs in four steps. In summary, tryptophan is first

hydroxylated to form 5-hydroxy-tryptophan (5HTP) by tryptophan-5-hydroxylase (TPOH). This product is subsequently decarboxylated to 5-hydroxy-L-trytamine (serotonin or 5-HT) under the catalytic action of aromatic amino acid decarboxylase (AADC). After that, serotonin is first acetylated to form N-acetylserotonin by AANAT and, finally, N-acetylserotonin is methylated to melatonin by N-acetyl-serotonin methyltransferase (ASMT). Both AANAT and ASMT are limiting enzymes in the synthesis of melatonin.

4.2 PINEAL MELATONIN

Pineal melatonin production is controlled by the SCN, which transforms the photoperiodic signal into an endocrine signal (Figure 4) (Acuña-Castroviejo et al., 2017). During the night, the polysynaptic pathway that establishes the SCN in the pineal gland gives rise to the stimulation of pinealocytes, which culminates in the expression of AANAT, one of the key enzymes in the production of melatonin (Klein, 2007). Once melatonin is synthesized in the pineal gland, it is rapidly released to the cerebrospinal fluid and blood due to its high diffusibility. As a result, the melatonin levels in both, the fluid and blood, have a circadian rhythm, which typically peaks at night (D. X. Tan et al., 2018). All these rhythmic variations, including endocrine and non-endocrine rhythms, during approximately 24 h have a chronobiotic impact on the organism.

Melatonin, in turn, affects the SCN and, therefore, regulates the circadian phase to maintain rhythmic stability, although the way in which this is done is unclear. Melatonin membrane receptors have been identified in the SCN of vertebrates, while signal transduction pathways are involved in both MT1 and MT2 (specific membrane receptors) in order to induce an increase in the expression of the two clock genes, *Per1*

and *Per2* (Kandalepas et al., 2016; D. X. Tan et al., 2018). Melatonin also has an acute inhibitory effect on neuronal firing through the inhibition of glutamatergic activity, independently of MT1 and MT2 (Escames et al., 2004). In addition, melatonin also binds other proteins such as calmodulin, calreticuline and quinone reductase II, leading to various regulatory effects (D. P. Cardinali et al., 2017; Targhazeh et al., 2022).

Melatonin is also able to change the phase in the expression of *Bmal1* and *Rev-erbα* (C. Liu et al., 1997; Vriend & Reiter, 2014). It has been hypothesized that melatonin maintains the regulation of the circadian machinery through the ubiquitin–proteasome system (explained in section 4.6.2). Thus, the effect of melatonin on the central pacemaker could act as a regulator of the circadian clock by regulating the correct time and rhythmic amplitude (Acuña-Castroviejo et al., 2017; Reiter et al., 2014).

Therefore, pineal melatonin is a chronobiotic molecule that plays a major role in the coordination of circadian rhythmicity. Functionally, pineal melatonin produces a host of effects that can be controlled by the SCN and may also have a direct impact on numerous peripheral organs. Thus, melatonin plays multiple roles in the complex circadian system by influencing the phases of the central pacemaker, as well as of peripheral oscillators (Figure 4). In particular, pineal melatonin is involved in sleep promotion (Hardeland et al., 2012). At the molecular level, increased melatonin levels are correlated with BMAL1 and CLOCK expression at night, which generates circadian rhythms through the transcriptional/translational feedback loop described above (Reppert & Weaver, 2002). Additionally, melatonin has been shown to modulate, either positively or negatively, the expression of most central oscillator genes. Nevertheless, the mechanisms by which melatonin participates in the expression of circadian clock components are unclear.

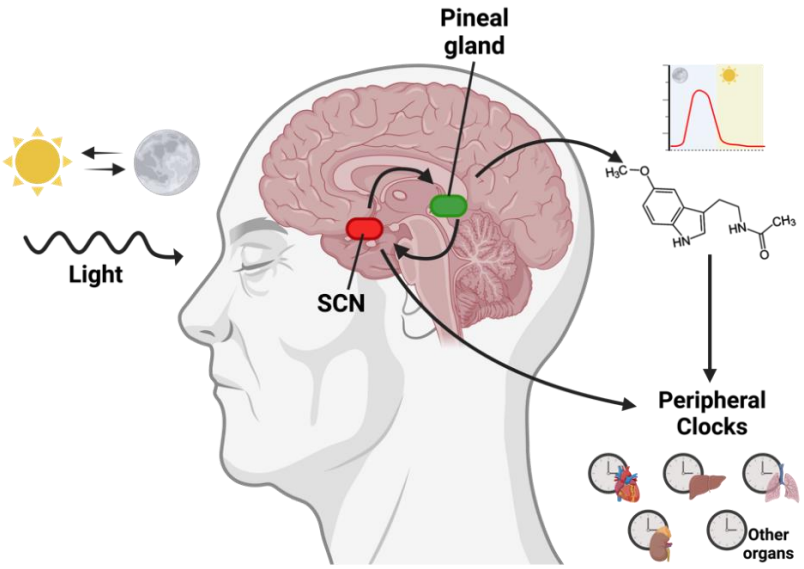

Figure 4. Schematic representation of the role of melatonin in the circadian system. In mammals, circadian rhythms are regulated by circadian clocks. The central clock is located in the SCN of the hypothalamus and controls pineal melatonin secretion in the absence of light, while melatonin also affects the SCN, which regulates chronobiotic activities. In peripheral tissues, clock genes are synchronized by the SCN and are also influenced by melatonin. Suprachiasmatic nucleus (SCN) (Rodríguez-Santana et al., 2023).

Alterations in pineal melatonin levels, caused mainly by light exposure at night, can disrupt the circadian system and have, consequently, been related to numerous pathologies, including carcinogenesis (Touitou et al., 2017).

As for its metabolism, circulating melatonin is mostly metabolized by the liver, although other organisms may also be involved. Melatonin is metabolized to 6-sulfatoxymelatonin (nearly 70%), principally in the liver via 6-hydroxylation and later through sulfate conjugation. Various minor metabolites include glucuronide conjugate, N1-acetyl-n2-formyl-5-methoxykynuramine (AFMK), and N1-acetyl-5-methoxykynuramine (AMK), which are excreted through urine (Tordjman et al., 2017). Interestingly, compared to the antioxidant properties of melatonin itself, melatonin metabolites have been described to have even greater antioxidant properties (D. X. Tan et al., 2015).

4.3 EXTRAPINEAL MELATONIN

The discovery of much higher concentrations of melatonin in gastrointestinal cells as well as others tissues allowed the discovery of extrapineal melatonin. It is currently known that most tissues produce melatonin and, except for some tissues such as the retina, the common absence of day:night variations in extrapineal melatonin synthesis suggests signaling pathways other than photoperiod that regulate the production of this hormone. Therefore, unlike pineal melatonin, extrapineal melatonin does not follow a circadian rhythm and, to date, it is not known whether its synthesis is controlled by clock genes (Acuña-Castroviejo et al., 2017; Venegas et al., 2012). Even in many tissues, extrapineal melatonin remains at concentrations above plasma concentrations for 24 hours and is generally not released into circulation, indicating that it acts in an autocrine or paracrine manner, protecting cells since extrapineal melatonin has antioxidants and antiflammatory actions and plays a key role in maintaining mitochondrial homeostasis.

4.4 INTERACTION OF MELATONIN IN MITOCHONDRIA

Mitochondria and chloroplasts are the main sources of free radicals in living organisms, as they are continuously generated during cellular respiration or photosynthesis (Hevia et al., 2015). Because of this, these organelles require strong protection against free radicals and associated oxidative stress. Therefore, melatonin is a critical molecule for preserving both mitochondrial and chloroplastic integrity and physiology. In fact, as has been thought for some years, Suofu and collaborators (Suofu et al., 2017) have described the synthesis of melatonin in the mitochondrial matrix that

is released by the organelle. Subsequently, this indolamine is bound to high-affinity MT1 located in the outer mitochondrial membrane with its ligand-binding domain facing the cytosol and whose signal transduction apparatus is located in the intermembrane space (Suofu et al., 2017). On the other hand, as already mentioned, thanks to its lipophilic nature, melatonin can cross both cellular and subcellular membranes, concentrating in different compartments, including the mitochondria (Venegas et al., 2012).

Therefore, melatonin is an excellent mitochondrial protector and regulates all mitochondrial bioenergetic functions, both *in vivo* and *in vitro*. One of its main functions is protection against mitochondrial oxidative stress and inhibition of apoptosis. The function of this hormone includes its ability to eliminate free radicals and to increase the activity and expression of antioxidant enzymes. An excessive accumulation of ROS produced by ETC contributes to the permeabilization of the outer mitochondrial membrane, controlled mainly by Bcl-2 family proteins, mediators of intrinsic apoptosis (Loureiro et al., 2015). The ability of melatonin to scavenge free radicals protects the mitochondria from oxidative damage and thereby reduces apoptosis in healthy cells. Moreover, melatonin decreases Bax as well as increases Blc2 inhibiting apoptosis.

Additionally, melatonin improves electron transport in the inner mitochondrial membrane by enhancing the ionic conductivity of the electron transport chain. As a result, melatonin increases the efficiency of the ETS and, in turn, reduces the production of free radicals and electron leakage that results from respiration by stimulating all the complexes of the mitochondrial respiratory chain that are involved in OxPhos (Acuna-Castroviejo, 2007; Florido, Rodriguez-Santana, et al., 2022). Melatonin increases the activity and expression of ETC complexes, mainly complexes I, III and IV, accelerating the flow of electrons and increasing ATP production. Melatonin also regulates the opening

of the mitochondrial permeability transition pore (mPTP), maintaining the membrane potential at optimal levels. More specifically, melatonin activates the PTPm and reduces the mitochondrial membrane potential. Nevertheles, to preserve membrane potential and prevent mitochondrial collapse, under conditions of oxidative stress where mPTP function is damaged, melatonin inhibits it (D. X. Tan et al., 2016). Therefore, under both favorable and unfavorable conditions, melatonin regulates the mitochondrial membrane potential, thus maintaining the function of the mitochondria to generate ATP.

Oxidative damage to mtDNA can produce specific mutations, deletions or point mutations within the mitochondrial genome which frequently lead to metabolic defects, cellular energy failures and ultimately disease. Therefore, the decrease in oxidative stress induced by melatonin, and several of its metabolites, prevents oxidative damage to mtDNA, both under normal and disease conditions in which great oxidative stress is generated (Prystowsky, 2015; Yamamoto & Mohanan, 2003).

Finally, melatonin plays a key role in mitochondrial dynamics, allowing the elimination of old and damaged mitochondria and the preservation of new healthy ones. In general, high concentration of melatonin produces a decrease in fission as well as a increase in mitochondrial fusion (Yang et al., 2023). For all these reasons, melatonin decreases the translocation to the mitochondria of proteins involved in mitochondrial fission such as Drp1 and Bax, and increases mitochondrial fusion proteins such as MFN1 and MFN2 and OPA1 (D. X. Tan et al., 2016). Additionally, melatonin also regulates mitophagy, a specific autophagy process in mitochondria that is essential for removing damaged mitochondria. It has been shown that melatonin can increase mitophagy and mitochondrial biogenesis (Proietti et al., 2017).

4.5 ONCOSTATICS EFFECTS OF MELATONIN

Since the middle of the last century, even before melatonin was isolated for the first time, several authors (Engel & Fischl, 1954; Sander & Schmid, 1952b, 1952a) described the beneficial effects of injecting melatonin extracts pineal in neoplastic patients. Since then, it has been shown that melatonin has important oncostatic effects (Fernandez-Gil et al., 2019; Florido, Martinez-Ruiz, et al., 2022; Guerra-Librero et al., 2021; Loureiro et al., 2015; Shen et al., 2018), while protecting healthy tissues from adverse effects caused by conventional cancer treatments (Fernández-Gil et al., 2017; Ortiz et al., 2015). Currently, the mechanisms of action of melatonin are not well understood, since almost as many mechanisms have been described as there are types of tumors. This could suggest the existence of a more primordial mechanism, underlying all the described phenomena. However, the oncostatic actions of melatonin in cancer can be classified into: antiproliferative and proapoptotic actions, stimulation of the immune system, anti and prooxidant actions, modulation of the expression of oncogenes, antiangiogenic effects and antimetastatic effects (D. Cardinali & Escames, 2016; Talib, 2018).

4.5.1 ANTIPROLIFERATIVE AND PROAPOPTOTIC ACTIONS OF MELATONIN

Tumor progression is closely related to the accelerated proliferation capacity of tumor cells. This is why certain cell cycle proteins have altered expression in tumor cells, including HIF-1, NF-κB (nuclear factor kappa light chain enhancer of activated B cells), PI3K/Akt, the insulin-like growth factor receptor (IGF-1R), dependent kinases of cyclins (CDKs) or the estrogen receptor (Talib, 2018). One of the main effects of melatonin on

tumor cells is its antiproliferative capacity, due to its modulating capacity of the cell cycle by inducing an arrest in the G1 phase (L. Liu et al., 2011; Nopparat et al., 2017). Furthermore, melatonin treatment has been shown to reduce HIF-1 levels, reduce the expression and translocation of NF-κB to the nucleus, inhibit the PI3K/Akt/mTOR pathway (Shen et al., 2018) as well as the expression of CDKs, producing a decrease in the proliferative capacity of tumor cells (Talib, 2018).

In addition, melatonin also has the ability to induce apoptosis by altering the Bax/Bcl-2 balance. This is because the permeabilization of the outer mitochondrial membrane is a point of no return and is regulated by proteins of the Bcl-2 family. Melatonin has been shown to inhibit Bcl-2 expression in various tumors (K. Chen et al., 2021a; Guerra-Librero et al., 2021; Xu et al., 2013). In this way, the release of cytochrome c to the cytosol would increase, thereby activating the caspases pathway. However, the sensitivity of tumor cells to melatonin-induced apoptosis is different in different cell lines (Shen et al., 2018).

4.5.2 ANTIOXIDANTS AND PROOXIDANTS EFFECTS

As we have described previously, melatonin has an antioxidant effect due to the ability of the molecule itself, as well as its metabolites, to scavenger free radicals, as well as to increase antioxidant enzymes activity and expression. Moreover, melatonin increases the efficiency of the ETC, reducing the escape of free radicals (Acuna-Castroviejo, 2007). Furthermore, the regulation of the redox state is crucial in tumor cells since an alteration of the redox balance and signaling is responsible for tumor progression and resistance to treatments (Panieri & Santoro, 2016). However, melatonin has a dual effect on the redox state of cells, because it protects normal cells

from oxidative damage while it functions as a pro-oxidant molecule in tumor cells by inducing the production of ROS (Florido, Rodriguez-Santana, et al., 2022). Various groups have, for many years, reported that high concentrations of melatonin can promote ROS generation, leading to cell death in a variety of cancers (Florido, Martinez-Ruiz, et al., 2022; Guerra-Librero et al., 2021; Laothong et al., 2015). Furthermore, research has shown that melatonin enhances the cytotoxic effects of chemotherapeutic drugs on cancer cells, suggesting an increase in the chemotherapeutic effect, thanks to an increase in oxidative stress (Fernandez-Gil et al., 2019; Shen et al., 2018).

However, the mechanisms by which this occurs in tumor cells are not entirely clear. What does seem to be clear is that the effect of melatonin, through its proven affinity for receptors, can induce oxidative stress directly by increasing ROS levels and indirectly by regulating the expression of various proteins involved in metabolic pathways, including SIRT1 and 3 (Florido, Rodriguez-Santana, et al., 2022). Furthermore, recently in our research group we have shown that melatonin at high doses, in HNSCC tumor cells, induces the production of ROS by reverse electron transport (RET) due to an increase in mitochondrial activity (Florido, Martinez-Ruiz, et al., 2022).

4.5.3 ANTIANGIOGENIC EFFECTS

Angiogenesis, the emergence of new blood vessels from preexisting vessels, is a tightly regulated process essential for tissue growth, development, and repair. However, in the context of cancer, it becomes a double-edged sword. Tumors induce angiogenesis to ensure a constant supply of nutrients and oxygen, facilitating their growth and progression. Therefore, angiogenesis is a hallmark feature of cancer, allowing tumors to grow, invade and metastasize, and, furthermore, dysregulation of angiogenesis is often

associated with poor prognosis in cancer patients. As a result of hypoxia, angiogenic mediators are activated in the central areas of a solid tumor, resulting in angiogenesis (Mehrzadi et al., 2021).

VEGF, a mitogenic, angiogenic mediator and potent stimulator of vascular permeability, is a potent enhancer of angiogenesis. Furthermore, hypoxia-inducible factor 1 (HIF-1), which is made up of HIF-1α and HIF-1β heterodimers, is another key factor in angiogenesis, which modulates the transcription of genes activated by hypoxia. Specifically, the α subunit of HIF-1β is constitutively expressed, however, HIF-1α is stabilized under hypoxic conditions and degraded under normoxic situations (Dudley & Griffioen, 2023).

Melatonin has been shown to have a regulatory role in the angiogenesis process. Melatonin promotes angiogenesis in certain physiological processes or skin lesions, while in a hypoxic environment, such as in tumors, melatonin suppresses neovascularization in tissues (Mehrzadi et al., 2021; Talib, 2018). There is numerous evidence that demonstrates an antiangiogenic effect of melatonin on tumor cells. In fact, there are various pathways through which this hormone acts, however, the inhibition of VEGF and HIF-1α transcription and translation is the most studied pathway (Mehrzadi et al., 2021).

4.5.4 STIMULATION OF THE IMMUNE SYSTEM

The immune system plays a crucial role in identifying and eliminating aberrant cells, including cancer cells. However, cancer often employs various strategies to evade immune detection and destruction, leading to disease progression. In this sense, melatonin can regulate the immune response against cancer cells (Carrillo-Vico et al.,

2013; Moslehi et al., 2022). On the one hand, melatonin increases the number and activity of natural killer cells due to a stimulation of the release of the interleukin IL-2 through the positive regulation of MT1 (Srinivasan et al., 2008). Furthermore, melatonin also enhances antigen presentation by macrophages to T lymphocytes, leading to the activation and proliferation of cytotoxic T cells (Lardone et al., 2009; Ren et al., 2017). Additionally, this hormone attenuates the activity of regulatory T cells and cancer-associated fibroblasts, cells responsible for facilitating the immune escape of cancer cells (D. Liu et al., 2021). Therefore, all these functions on the immune system allow the recognition and elimination of tumor cells (Mortezaee et al., 2019).

4.5.5 ANTIMETASTATIC EFFECTS

The spread of cancer cells from the primary tumor to distant sites in the body remains a formidable challenge in cancer treatment. Metastasis is a complex process that consists of several stages: cancer cells must escape from the primary site, survive in the circulation and develop in distant tissues. Regarding this, melatonin is also capable of interfering in several steps necessary for the development of metastasis, such as the loss of cell-cell contact, tissue invasion and extravasation (Su et al., 2017). On the one hand, melatonin increases the expression of E-cadherin or occludin, proteins necessary for the formation of tight junctions in cell-cell adhesion. Furthermore, melatonin inhibits the epithelial-mesenchymal transition, a process by which tumor cells lose their adhesion to neighboring cells and become migratory by altering the signaling of the NF-kB pathway (Su et al., 2017; S. Wu et al., 2016).

On the other hand, the inhibitory capacity of melatonin on the induction and catalytic activity of MMP-9, a matrix metalloproteinase, responsible for the degradation

of the extracellular matrix, has been demonstrated (Lin et al., 2016; Rudra et al., 2013). We must even add the antiangiogenic effects mentioned above. All this evidence and exemplifies the complex mechanisms by which melatonin is capable of reducing metastasis.

4.6 MELATONIN AND CLOCK GENES IN CANCER

Numerous studies have demonstrated the relationship between disrupted clock genes, cancer and decreased levels of melatonin (Stevens, 2005; Zhou et al., 2022). Moreover, the suppression of melatonin production is associated with an increased incidence of cancer (Schernhammer et al., 2006; Touitou et al., 2017), which could be correlated with a loss in the regulation of the circadian machinery. Additionally, a large number of studies have described numerous oncostatic effects of this hormone (Bhattacharya et al., 2019; Y. Li et al., 2017; Targhazeh et al., 2022), however few have focused on the relationship of melatonin on circadian rhythms in cancer.

4.6.1 MELATONIN AND SIRTUIN 1

Melatonin also modulates clock genes through sirtuin 1 (SIRT1) (Bonomini et al., 2018; Tajes et al., 2009)(Figure 5). Already in 2009, Jung-Hynes B and Ahmad N (Jung-Hynes & Ahmad, 2009) proposed that the inhibition of SIRT1 could be the key to the regulation of the circadian machinery. This regulation occurs by controlling the acetylation levels of both BMAL1 and the circadian repressor PER2, thus boosting PER2 degradation and circadian cycle rhythmicity (Figure 5). SIRT1, which is associated with CLOCK, is recruited to the CLOCK:BMAL1 chromatin complex at circadian promoters, thus facilitating the circadian expression of numerous genes, including *nampt*.

Therefore, a decrease in NAMPT levels and, chronically, an increase in NAD+ are related to abnormal circadian behavior and metabolism (Imai, 2010; Xie et al., 2020).

Sirt1, which is overexpressed in tumor cells, is correlated with the silencing of tumor suppressor genes and with cancer resistance to chemotherapy. Interestingly, melatonin has a dual effect on SIRT1 expression. In normal cells, melatonin decreases ROS production and regulates cell homeostasis. However, in tumor cells, melatonin downregulates SIRT1, which results in proapoptotic and prooxidant activity (Florido, Rodriguez-Santana, et al., 2022). Specifically, in human osteosarcoma, melatonin has been shown to inhibit SIRT1, resulting in increased prooxidant and antitumor activity (Cheng et al., 2013). The upregulation of SIRT1 by SRT1720 (a known SIRT1 activator) attenuates melatonin's antioxidant and antitumor activity, indicating that its induction of ROS production in tumor cells is activated by SIRT1 (Proietti et al., 2014). In addition, in MCF7, Proietti et al.S 2014 have described how the inhibition of SIRT1 levels by melatonin produces the downregulation of the MDM2 pathway, a ubiquitin protein ligase, which enhances p53 acetylation and, therefore, p53 activity. Although the mechanisms by which melatonin affects SIRT1 are still not fully understood, in 2009, Hill et al. (Hill et al., 2009) were the first to suggest that melatonin, through the MT1 receptor, blocks the transcription of RORα, thus blocking the activation of Bmal1 expression and, therefore, of Sirt1 (Hill et al., 2009).

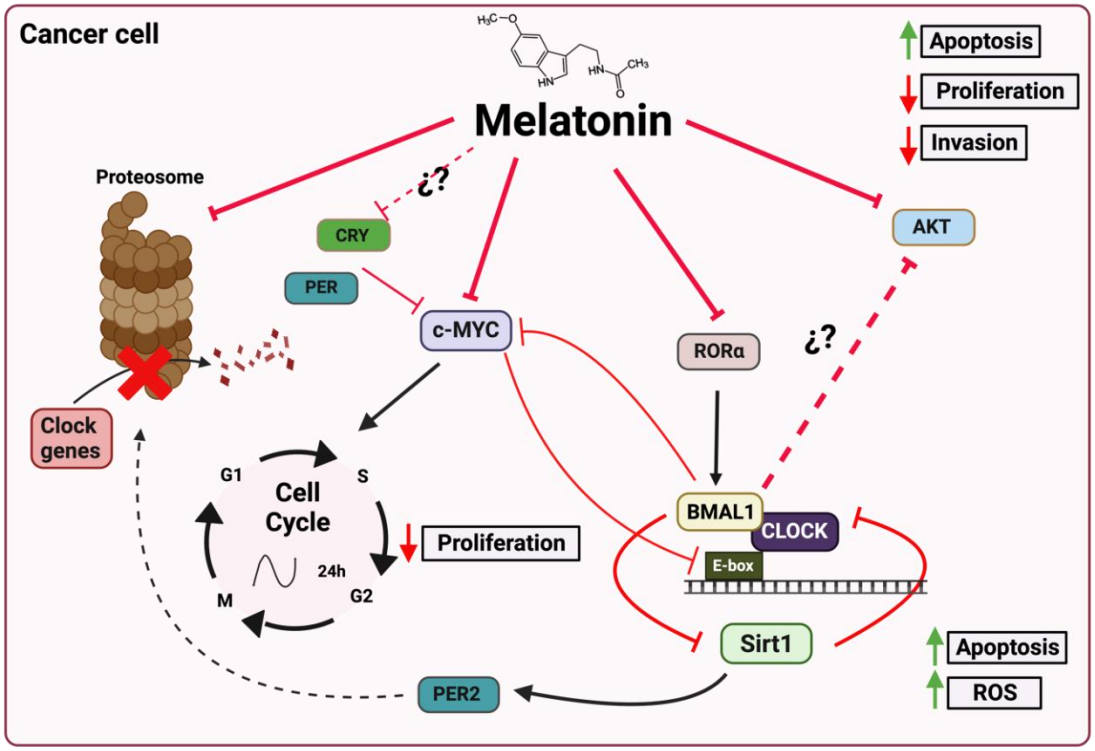

Figure 5. Proposed mechanisms by which melatonin affects the circadian machinery of cancer cells. By inhibiting the proteasome, melatonin can act post-translationally to regulate the circadian clock by stabilizing proteins, depending on the time of day and the levels of this hormone. Melatonin, together with BMAL1, PER and CRY, represses c-Myc, which arrests the cell cycle, thus decreasing cell proliferation. In addition, c-MYC binds to the E-box region and regulates the expression of certain clock genes. Through the MT1 receptor, melatonin blocks the transcription of RORα and also the activation of BMAL1 expression, as well as of Sirt1, with consequent proapoptotic and prooxidant effects. In addition, SIRT1 regulates BMAL1 by controlling acetylation levels and PER2 promoting its degradation. Melatonin inhibits the AKT pathway, which may occur via BMAL1. The inhibition of this pathway causes an increase in apoptosis and a decrease in cell proliferation and invasion. Brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (Bmal1); circadian locomotor output cycles kaput (Clock); Chryptochrome (Cry); Period (Per); orphan retinoic acid receptor-related alpha (RORα); reactive oxygen species (ROS); sirtuin 1 (Sirt1) (Rodríguez-Santana et al., 2023).

4.6.2 MELATONIN AND THE UBIQUITIN–PROTEASOME SYSTEM

Several mechanisms have been described to explain the oncostatic effects of melatonin through its effect on clock genes. One of these mechanisms, mentioned above, is the ubiquitin–proteasome system, which enables the degradation of proteins such as BMAL1, REV-ERVα, CRY and PER to be regulated. However, while there is no

direct evidence to show that melatonin directly inhibits proteasomes in the SCN, melatonin clearly may interfere with the activity of the ubiquitin–proteasome system in a variety of tissues, including the SCN (Park et al., 2014; Vriend & Reiter, 2014). Vriend and Reiter (Vriend & Reiter, 2015) have suggested that melatonin inhibits the proteasome, which provides selective stability to proteins during periods of elevated melatonin levels. This effect of melatonin on the proteasome results in increased levels of CRY, PER and REV-ERBα, which, in turn, regulate the transcription of Bmal1. However, the authors highlight the fact that BMAL1 is closely associated with the inhibition of melatonin in the proteasome, whose expression coincides with the nocturnal increase of melatonin (Vriend & Reiter, 2015). Moreover, it has been shown that melatonin inhibits the proteasomes of renal cancer cells (Figure 5) (Park et al., 2014).

Currently, it is known that clock genes are closely linked to cell cycle-related genes, whose levels are controlled by the proteasome (Smith et al., 2007). Thus, given that melatonin acts as a proteasome inhibitor, its effects on the inhibition of cyclin D1 and E, CDK2, CDK4 and c-Myc expression in brain cancer stem cells (Lee et al., 2018) or on the reduction of cyclin D1 and E expression in breast cancer cells (Cini et al., 2005; Rögelsperger et al., 2011) could be closely linked. In addition, in different cancer cells, melatonin and the proteasome inhibitor bortezomib have been shown to have similar proapoptotic effects, indirectly interacting with the levels of p53, Bcl-2, BAX, p21 and NF-kB, through the ubiquitin–proteasome system. Furthermore, other studies have reported that melatonin increases apoptosis due to an increase in the Bcl-2-interacting mediator (BIM) of cell death via the inhibition of proteasome activity (Park et al., 2014).

4.6.3 MELATONIN AND C-MYC

Another important connection between the oncostatic effects of melatonin and circadian machinery is its ability to reduce the levels of c-Myc, leading to antitumor and antiproliferative effects (Ferreira et al., 2020; Lee et al., 2018). c-Myc, which is involved in oncogenesis processes, is overexpressed in many cancers. This is explained by the abnormal c-Myc levels, which enable cells with damaged DNA to pass through cell cycle checkpoints, thus contributing to the genesis of many cancers in humans (Fagundo-Rivera et al., 2020; Z. Liu et al., 2020). In recent years, this oncogene has been found to control and, in turn, to be controlled by the circadian machinery (Z. Liu et al., 2020). Although the circadian connection is currently not entirely clear, MYC is known to heterodimerize with its partner MAX in order to bind to E-box sequences (CACGTG) at cognate promoters for the purposes of gene regulation, such as the CLOCK-BMAL1 heterodimer (Wolf et al., 2015). MYC has also been shown to be regulated by CRY and PER proteins (Fu et al., 2002; Hua et al., 2006). Furthermore, downregulation of BMAL1 is associated with high MYC expression levels in a variety of tumors, a phenomenon associated with poor clinical outcomes (Altman et al., 2015). Given the multi-faceted involvement, such as increased cell cycle proliferation and increased glycolysis, of c-Myc in oncogenesis, as well as the oncostatic effects of melatonin by reducing c-Myc expression, we suggest that there is a clear link between this hormone and c-Myc, which needs to be studied in more depth (Figure 5).

4.6.4 MELATONIN AND AKT

The Akt signaling pathway is involved in the inhibition of cell apoptosis and in stimulating cell proliferation following the activation of Akt/PKB, a serine/threonine

kinase. In xenografts of breast cancer, the tumor growth rate and elevated levels of AKT and 3-phosphoinositide-dependent kinase-1 (PDK-1), an AKT stimulator, correlated with night-time light exposure and reduced levels of melatonin (Dauchy et al., 2014). In addition, numerous studies have reported the oncostatic effects of exogenous melatonin due to AKT downregulation (K. Chen et al., 2021b; Phiboonchaiyanan et al., 2021; Shen et al., 2018). There is consistent evidence that shows that melatonin may affect the phosphorylation of AKT, PI3K and GSK3β due to the regulation of Bmal1. In 2013, Jung, C. H. et al. (Jung et al., 2013) published a study that showed that the activation of the PI3K-Akt-MMP-2 pathway depends on BMAL1. Specifically, they demonstrated how the inhibition of BMAL1 increased the levels of PI3K activity, AKT phosphorylation, MMP-2 protein and, consequently, cell invasion. An interesting connection has thus been established between the inhibitory effect of melatonin on the AKT pathway and its ability to regulate Bmal1 (Figure 5).

HYPHOTESIS AND OBJETIVES

Considering the increase in scientific evidence that relates chronodisruption to carcinogenesis and how more and more research demonstrates alterations in the expression of clock genes in tumors, there is special interest in the regulation of these genes as a possible therapeutic target.

In this way, we currently know that melatonin is mainly responsible for the regulation of circadian rhythms. Furthermore, we know that many of the oncostatic effects of this hormone are due to a direct effect on the mitochondria. In this way, clock genes are closely related to mitochondrial metabolism and, recently, it has been shown that mitochondrial activity itself regulates clock genes, giving rise to a complex feedback loop.

Based on the above, in this study, we hypothesized that melatonin leads to numerous oncostatic effects in HNSCC by resynchronizing the deregulated circadian machinery of these cancer cells by focusing on the interplay between mitochondrial function and the functioning of the biological clock.

To verify this hypothesis, the objectives of this study were:

Principal objective:

To study the connection between clock gene desynchronization and mitochondrial dysfunction in head and neck squamous cancer cells, and to analyze the effects of melatonin in these cells.

Specifics objetives:

- To study the expression of clock genes in SCC-9 and Cal-27 tumor cells treated with and without melatonin.

- To analyze the connection between clock gene desynchronization and mitochondrial function in SCC-9 and Cal-27 tumor cells treated with and without melatonin.

- To evaluate the effects of melatonin in Cal-27 and SCC9 cells that have inhibited BMAL1 using SiRNA.

MATERIALS AND METHODS

1. Cells and treatments

Squamous cell carcinoma cells, Cal-27 and SCC9, was obteined from the Cell Bank of the Scientific Instrumentation Centre of the University of Granada (ATCC: CRL2095 and CRL1629, respectively). Cal-27 cells were maintained in Dulbecco's modified Eagle's médium High Glucose (DEMEM; DMEM-HHSTA, Capricorn Scientific GmbH, Ebsdorfergrund, Germany) supplemented with 10% fetal bovine serum (FBS; 16000044; ThermoFisher Scientific, Waltham, MA, USA), 2% anti-biotic/antimycotic solution (15240062; ThermoFisher Scientific) and 1 mM sodium pyruvate (S8636-100ML; Sigma, Madrid, Spain). SCC9 cells were grown in DMEM-F12 (11320033; 1:1; ThermoFisher Scientific) Nutrient Mixture Ham medium containing 10% FBS, 2 mM L-glutamine (25030081; ThermoFisher Scientific), 0.4 µg/mL hydrocortisone (H0888; Sigma), 2% antibiotic/antimycotic solution, and 0.5 mM sodium pyruvate. Both cell lines were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air. The medium was changed every 2-3 days until they reached 70-80% confluency, at which point subculture was performed. Moreover, mycoplasma test was performed to confirm that Cal-27 and SCC9 cells using a PCR detection kit following the manufacturer's instructions (25235, LiliF Diagnostics, Burlington, MA, USA).

Melatonin (Fagron Ibérica S.A.U., Zaragoza, Spain) stock solution was prepared in 15% propylene glycol (24414.296; VWR, Radnor, PA, USA) in phosphate buffer solution (PBS; 14190-094; Life Technologies, Carlsbad, CA, USA) and filter-sterilized through a 0.2 µm-pore filter (Sartorius Biotech GmbH, Gottingen, Germany). Cells were grown to 60–70% confluence and serum starved for 48 h. They were then treated with different melatonin concentrations (10 nM, 100 µM, 500 µM and 1000 µM). The

cells were harvested and assayed at the different time points indicated in the figures/texts. Vehicle was added to the control group.

2. Gene expression analysis

2.1 RNA extraction

Total RNA from Cal-27 and SCC9 was isolated from frozen cell pellets using NZY Total RNA isolation kit (MB13402, Nzytech gene & expression, Lisbon, Portugal) following the manufacturer's instructions. RNA was quantified in a Nano Drop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and its integrity was confirmed by 2% agarose gel electrophoresis. RNA was aliquoted and stored at -80 °C for its later reverse transcription into cDNA.

2.2 Reverse transcription reaction

RNA was reverse transcribed to cDNA from 400 ng total RNA with NZY First-Strand cDNA Synthesis kit (MB12501, Nzytech gene & expression, Lisbon, Portugal). The samples were loaded into a Techne Thermal Cycle / PCR model FTGene2D (Techne, Cambridge, UK). The reverse transcription reaction conditions are specified in Table 1. After finishing the program, the cDNA obtained was aliquoted and stored at -20 °C until later use

Table 1. Conditions programmed into the thermal cycler for reverse transcription.

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	50	85	4
Time (min)	10	30	5	∞

2.3 Real-time reverse transcription polymerase chain reaction (RT-PCR)

Table 2. List of primers used in RT-PCR assay.

Gene Symbol	Gene Description	Forward primer	Reverse primer
Bmal1	Brain and muscle ARNT (aryl hydrocarbon receptor nuclear translocator)-like 1	ATCCTCAACTACAGCCAGAATG	TCGTGCTCCAGAACATAATCG
Clock	Circadian Locomotor Output Cycles Kaput	ACGACGAGAACTTGGCATTG	TCCGAGAAGAGGCAGAAGG
Per2	Perio circadian clock 2	CCCTTCCGCATGACGCCCTACCTG	GACCGCCCTTTCATCCACATCCTG
Cry1	Cryptochrome circadian regulator 1	GCTTGCTTCCTGACACGAG	GACAGCCACATCCAACTTCC
Rev-Erbα	Reverse strand of protein ERB alpha	TCCCCCAGCAAGAGCACCAGCAACAT	CCCGAGGCAACGTCCCCACAC
RORα	Retinoica cid-related orphan receptor alpha	ATAATCGGTGACTGGTGTGC	GCTATTGTTGCTACTGCTATGG
Sirt1	Sirtuin 1	ACAGGTTGCGGGAATCCAAA	GTTCATCAGCTGGGCACCTA
Sirt3	Sirtuin 3	ACCCAGTGGCATTCCAGAC	GGCTTGGGGTTGTGAAAGAAG
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	GTACTACACTGAATTCACCCCCACTG	TGCGGCATCTTCAAACCTCCAT

Amplification was performed by quantitative real-time polymerase chain reaction (RT-PCR) in a Stratagene Mx3005P QPCR System (Agilent Technologies, Madrid, Spain) with NZY Supreme qPCR Green Master Mix (2x) (MB41902, Nzytech gene & expression, Lisbon, Portugal). Primers were designed using the Beacon Dedigner software 4.0 (Premier Biosoft Inc., Palo Alto, CA, USA) and they are showed in the Table 2. Thermal profile of RT-PCR is indicated in Table 3. Expression levels of the target gene were normalized using the housekeeping control gene *Gapdh*. Template-free (water) sample was used as a negative control. mRNA amount of each target gene relative to *Gapdh* was calculated through the comparative Ct method (i.e. the $2^{(-\Delta\Delta Ct)}$ method).

Table 3. RT-PCR cycle program.

Cycles	Temperature (°C)	Time	Step
1	95	2 min	Polymerase activation
40	95	5 sec	Denaturation
	60	30 sec	Annealing/extension

3. BMAL1-specific siRNA transfection

To study the role of Bmal1 following melatonin treatment, we decided to silence its expression using siRNA. To do this, we performed a transient transfection that allowed us to silence the target gene by binding the siRNA to the mRNA.

Procedure

BMAL1-specific siRNA was purchased from Sigma-Aldrich (Mission Pre-designed siRNA2D). Cal-27 and SCC9 cells were seeded on 24 well plates at 60–70% confluence

and were transiently transfected with the BMAL1-specific siRNA diluted in Opti-MEM using Lipofectamine™ RNAiMAX Transfection Reagent (13778075, ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. After 24 h of incubation at 37 °C, the transfection medium was replaced with complete medium containing 10% FBS, and the experiments were conducted on the basis of the treatments carried out in the previous experiments.

4. Measurement of mitochondrial respiration

Because mitochondrial respiration is more physiologically relevant than isolated mitochondria since they preserve their essential interactions with other intracellular systems, we decided to investigate mitochondrial respiration using Clark electrodes in Cal-27 cells. These data allow us to clarify a correlation between mitochondrial function and the expression of clock genes in HNSCC.

4.1 Measurement of oxygen consumption rate by Clark electrode

This electrode is known as "Clark Type" after their inventor, Leland Clark (Clark et al., 1953). The Clark electrode consists of an anode and cathode in contact with an electrolyte solution. It is covered at the tip by a semi-permeable membrane which is permeable to gases but not contaminants and reducible ions of the sample. The cathode is in a glass envelope in the body of the electrode. The anode consists of silver/silver chloride (Ag/AgCl), and a potassium chloride (KCl) electrolyte reservoir, which provide electrons for the cathodic reaction. With application of a polarization voltage (usually 0.6 V), a current is obtained as an amperometric signal, which is converted to a voltage.

Procedure

The oxygen consumption rate (OCR) was measured by a high resolution oxymeter with a controlled oxygraph vessel equipped with a Clark oxygen electrode at 37°C (Oxygraph-2 k, Oroboros Instruments). Cal-27 and SCC9 intact cells, at a density of $1 \cdot 10^6$ cells/ml, were added to the oxygraph chamber containing DMEM-F12 medium at a final volume of 500 µL. After achieving a stationary endogenous substrate-sustained resting OCR, this rate was corrected for 2 µM antimycin A plus 2 µM rotenone-insensitive respiration. Results were normalized by cell number, and viability was determined using trypan blue staining.

5. Cell proliferation assay

DNA content is a simple and accurate method of measuring cell number since it remains constant for a given cell line or cell type. For that reason, cell proliferation was evaluated using the CyQUANT® assay (C7026, ThermoFisher Scientific). The main component of the CyQUANT® Cell Proliferation Assay Kit is CyQUANT® GR, a proprietary dye that shows strong fluorescence enhancement when bound to nucleic acids. With this dye, the DNA content of a sample can be measured and compared to standards by directly quantifying the entire cell population within a wide linear detection range.

Procedure

Cells were seeded in 96-well plates at a density of 8,000 cells per well and treated as previously described. After treatment, cells were washed with PBS and subsequently stored at -80 °C. On the day of the experiment, after thawing the plates at room

temperature, we then added 200 µL of CyQUANT® GR cell lysis dye/buffer to each sample well. The samples were incubated for 2 to 5 minutes at room temperature, protected from light. Fluorescence was measured (excitation, 480 nm; emission, 520 nm) using a microplate fluorescence reader FLx800 (Bio-Tek Instruments, Inc., Winooski, VT, USA) and compared with a standard curve for cell number determination.

6. Statisticals analysis

Data are expressed as the mean standard error of the mean (SEM) of a minimum of three independent expriments. Statistical analyses were carried out using GraphPad Prism 8.0.1 software (GraphPad Software, San Diego, CA, USA). Unpaired Student's t-tests were used to compare differences between experimental groups and their respective untreated controls. The values were found to be significantly different when $p<0.05$.

For circadian rhythm studies, Cosinor analysis (Nelson et al., 1979) was performed with R software 2020 (RStudio, Inc., Boston, MA, USA). For this purpose, a rhythmicity analysis of the experimental data was carried out using the non-linear Cosinor regression tool CircaCompare which allowed us to compare the parameters between two groups (Parsons et al., 2020). Circadian rhythm detection was considered statistically at $p<0.05$. An average level of three parameters is included in rhythm characterization, calculated with 95% confidence limits: the mesor (an acronym for midline estimating statistics of rhythm), the amplitude (half the difference between the minimum and maximum of the fitted cosine function), and the acrophase (the timing of the cosine maximum).

RESULTS

Part of the results shown in this section are included in the following article:

Rodríguez-Santana C, López-Rodríguez A, Martínez-Ruiz L, Florido J, Olga C, Capitanio N, Ramirez-Casas Y, Acuña-Castroviejo D, Escames G. The Relationship between Clock Genes, Sirtuin 1, and Mitochondrial Activity in Head and Neck Squamous Cell Cancer: Effects of Melatonin Treatment. doi: 10.3390/ijms241915030.

International Journal of Molecular Science, 2023

1. High concentrations of melatonin significantly affected the expression of clock genes *Bmal1* and *Per2* in HNSCC cells

Studies have shown that certain circadian genes are altered in some cancers (Benna et al., 2017; Kettner et al., 2014; Mocellin et al., 2018). Moreover, previous studies have demonstrated that only high doses of melatonin lead to HNSCC apoptosis due to excessive ROS production (Florido, Martinez-Ruiz, et al., 2022). Previously, we performed a dose response study, and our data suggested that there is a correlation between the cell content of melatonin and its apoptotic effects, thus supporting the notion that high concentrations of melatonin in cancer cells are required to its cytotoxic effects (Florido, Martinez-Ruiz, et al., 2022). However, the expression patterns of clock genes have not been studied at high doses of melatonin. Therefore, we first analyzed the expression of the clock genes *Bmal1* and *Per2* in Cal-27 and SCC9 cells treated with vehicle (control) or melatonin (500 and 1000 μM) at different time points during a period of 24 h. In addition, we established another experimental group, in which Cal-27 and SCC9 cells in culture were exposed to the well-established serum shock protocol to reset the clockwork machinery (Balsalobre et al., 1998), thus facilitating the homogeneity of clock gene expression once the repressive condition is removed (Fig. 6). The statistical data relating to the cosinor analysis of clock gene rhythms, including their significance, acrophase and amplitude, are shown in Table 4.

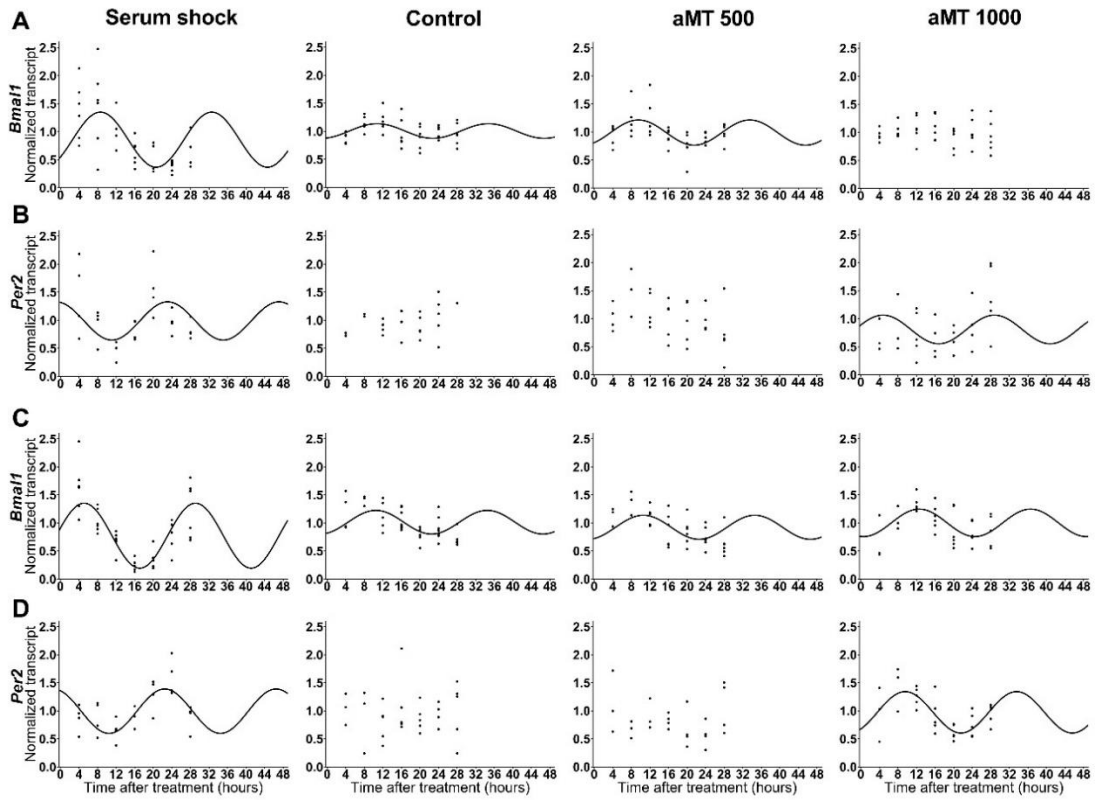

Figure 6. Relative expression of clock genes in HNSCC cell lines Cal-27 (A and B) and SCC9 (C and D): (A and C) *Bmal1* and (B nd D) *Per2* after serum shock, control and aMT (500 and 1000 µM) treatments; n= 3-6 independent experiments. The best cosinor fit is shown as a continuous line at a time of 48 h.

Table 4. Cosinor analysis of relative expression of the clock genes *Bmal1* and *Per2* in HNSCC cell lines Cal-27 and SCC9 after serum shock, control (vehicle) and aMT treatments (500 and 1000 µM).

Cell line	Gene	Treatment	Presence of rhythmicity (p-value)	Acrophase	Amplitude
Cal-27	***Bmal1***	Serum shock	<0.001	8.570589	0.492039
		Control	<0.01	10.76038	0.13079
		aMT 500 µM	<0.001	9.514851	0.225551
		aMT 1000 µM	ns	-	-
	Per2	Serum shock	<0.01	23.05335	0.341575
		Control	ns	-	-
		aMT 500 µM	ns	-	-
		aMT 1000 µM	<0.05	4.777426	0.256121
SCC9	***Bmal1***	Serum shock	<0.001	5.105071	0.580572
		Control	<0.001	10.4789	0.211801
		aMT 500 µM	<0.01	10.58747	0.215423
		aMT 1000 µM	<0.001	12.57654	0.245783
	Per2	Serum shock	<0.001	22.4778	0.396895
		Control	ns	-	-
		aMT 500 µM	ns	-	-
		aMT 1000 µM	<0.001	9.562281	0.369996

Regarding *Bmal1*, both cell lines showed a circadian basal expression in the control group. However, following synchronization with serum shock, the expression of *Bmal1* showed significant differences with respect to the control. Cal-27 cells displayed a broader amplitude, while SCC9 also showed an earlier acrophase. Cells treated with melatonin did not show differences with respect to the control except at a dosage of 1000 µM, at which melatonin induced a loss of circadian rhythm in *Bmal1* (Figure 7 and Table 5).

The rhythm of the other regulator gene *Per2* was also detected after serum shock in both cell lines, Cal-27 and SCC9. Nevertheless, no circadian rhythm was detected in the control group, without serum shock. However, 1000 µM melatonin induced a

circadian rhythmicity with respect to *Per2* in both cell lines. In addition, the comparison between serum shock and melatonin 1000 µM showed a significant difference in the acrophase (Figure 7 and Table 5). The expression of *Per2* has an inhibitory effect on *Bmal1*. This would explain the effect observed at aMT 1000 µM on Cal-27 (Fig.6A) upon induction of a circadian rhythm by *Per2* at aMT 1000 µM (Fig. 6B). However, this does not occur in SCC9, further suggesting a clock gene-independent function of *Per2.*

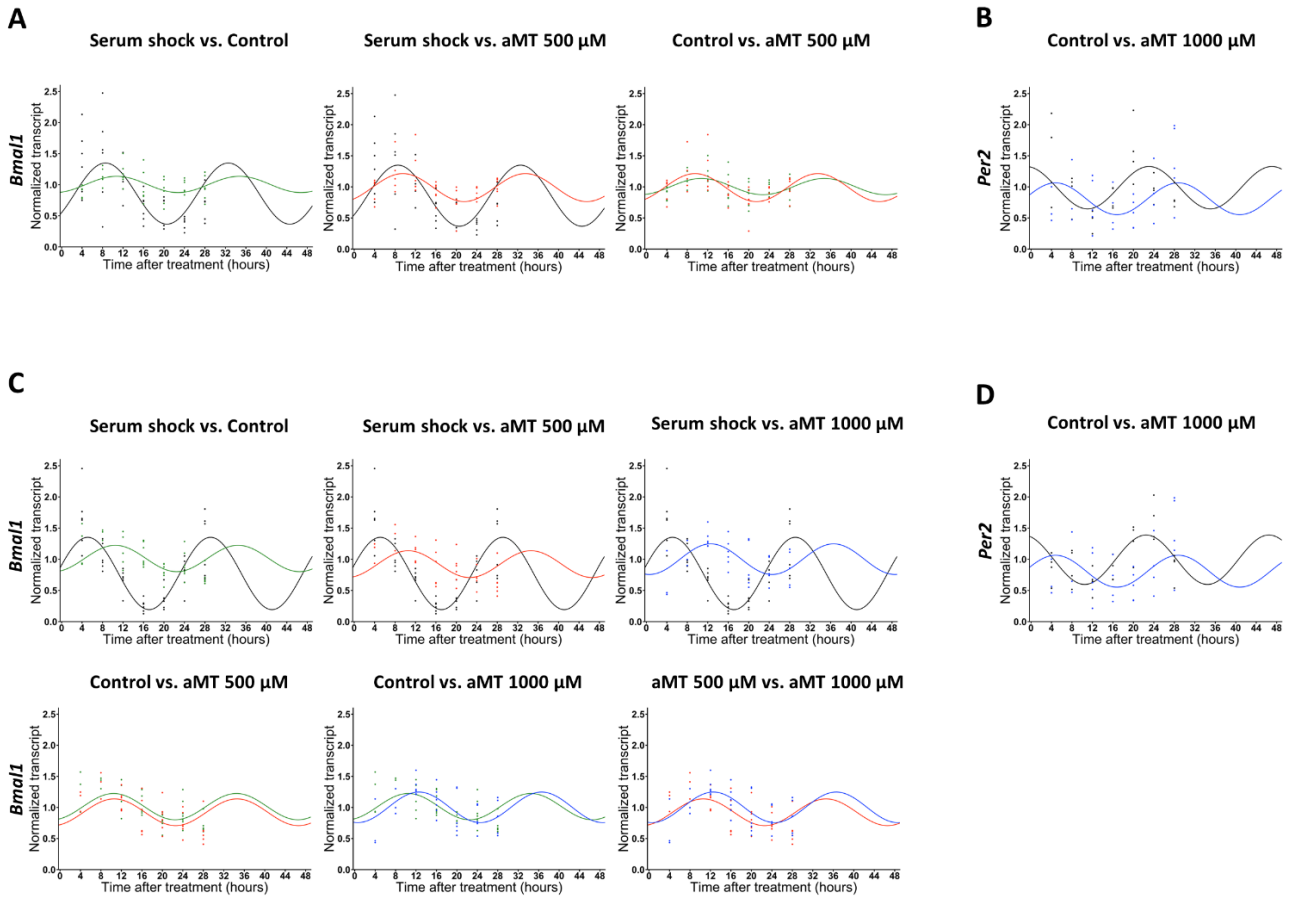

Figure 7. Cal-27 (A and B) and SCC9 (C and D) cosinor's best fits comparison of gene expression between different experimental groups. (A and C) *Bmal1* and (B nd D) *Per2*. Serum shock (black), control (green) and aMT 500 µM (red) and 1000 µM (blue) treatments; n= 3-6 independent experiments.

Table 5. Circadian rhythm comparison of relative expression of clock genes *Bmal1* and *Per2* in HNSCC cell lines Cal-27 and SCC9. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Cell line	Gene	Comparison	P-value for acrophase difference	P-value for amplitude difference
Cal-27	***Bmal1***	Serum Shock vs. Control	ns (0.328288)	** (0.002598)
		Serum shock vs. aMT 500 µM	ns (0.506968)	* (0.029825)
		Control vs. aMT 500 µM	ns (0.407893)	ns (0.182816)
	Per2	Serum shock vs. aMT 1000 µM	ns (0.012848)	ns (0.613012)
SCC9	***Bmal1***	Serum shock vs. Control	*** (2.95E-05)	*** (0.000234)
		Serum shock vs. aMT 500 µM	*** (7.57E-05)	*** (0.000461)
		Serum shock vs. aMT 1000 µM	*** (3.8E-08)	*** (0.00063)
		Control vs. aMT 500 µM	ns (0.940019)	ns (0.968606)
		Control vs. aMT 1000 µM	ns (0.125217)	ns (0.693895)
		aMT 500 µM vs. aMT 1000 µM	ns (0.176089)	ns (0.738686)
	Per2	Serum shock vs. aMT 1000 µM	ns (1.25E-15)	ns (0.653382)

2. Expression of *Bmal1* is not affected by low doses of melatonin in HNSCC cells

Since melatonin causes a resynchronization of the various clock genes in non-cancer cells at low doses, we analyzed the effects of melatonin at 10 nM and 100 µM on Cal-27 cells (Figure 8 and Table 6). The results did not show significant differences with respect to the control group, as are detailed in Figure 9 and Table 7. Therefore, we concluded that low doses of melatonin did not have any significant effect on the circadian rhythmicity of *Bmal1* in HNSCC cells.

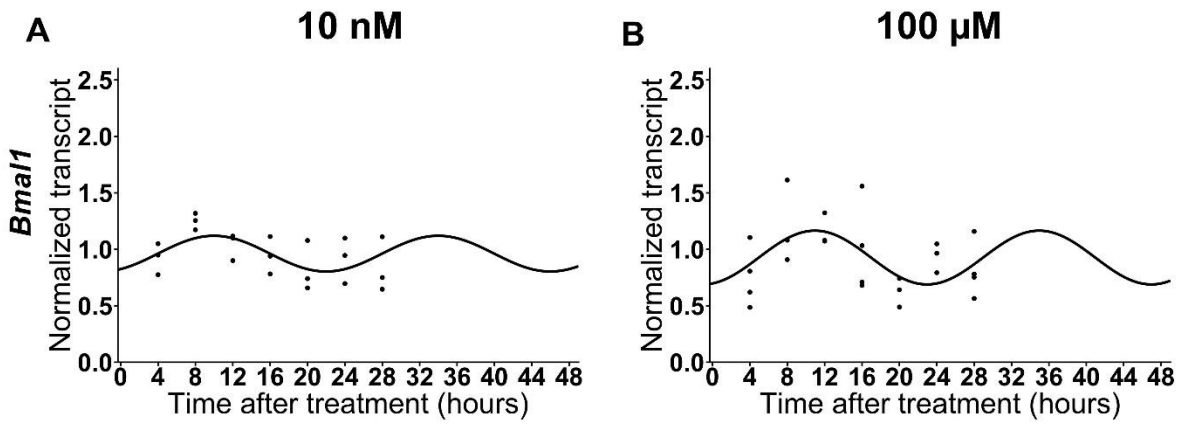

Figure 8. Relative expression of Bmal1 in Cal-27 cells with 10 nM aMT (A) and 100 µM aMT (B) treatments; n= 3-4 independent experiments. The best cosinor fit is shown as a continuous line at a time of 48 h.

Table 6. Cosinor analysis of relative expression of the clock gene *Bmal1* in HNSCC cell line Cal-27 after aMT treatments (10 nM and 100 µM).

Treatment	Presence of rhythmicity (p-value)	Acrophase	Amplitude
aMT 10 nM	<0.05	9.981817	0.158323
aMT 100 µM	<0.05	10.95637	0.238408

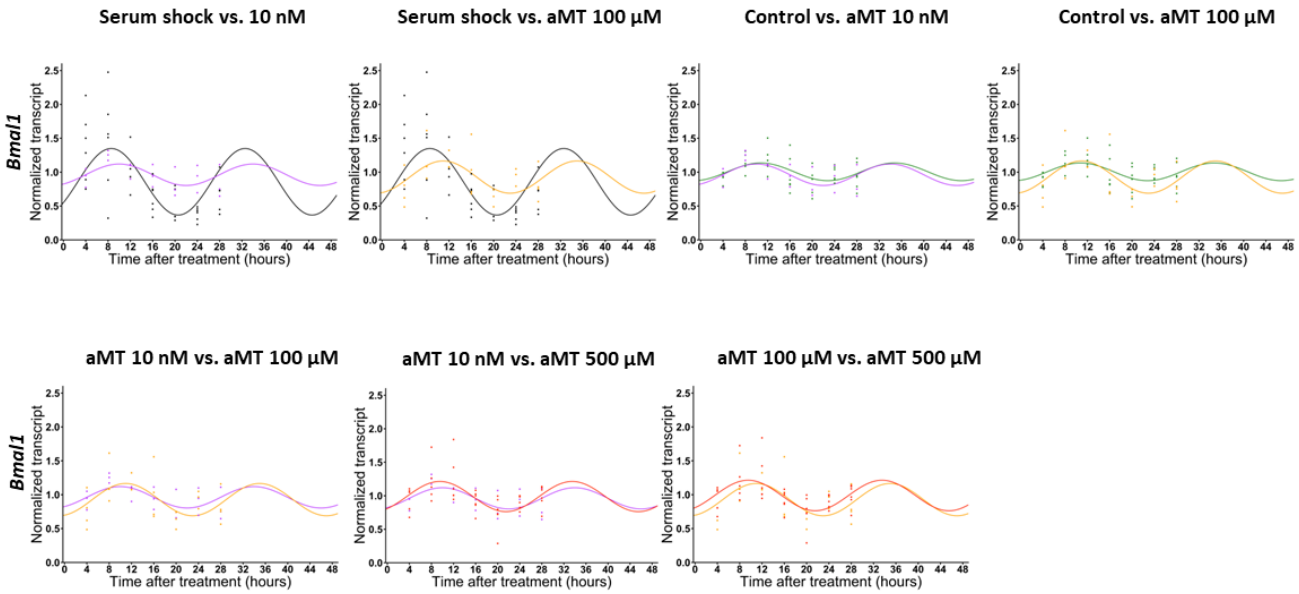

Figure 9. Cal-27 cosinor's best fits comparison of *Bmal1* between different experimental groups. Serum shock (black), control (green) and aMT 10 nM (purple), 100 µM (orange), 500 µM (red) and 1000 µM (blue) treatments; n= 3-6 independent experiments.

Table 7. Circadian rhythm comparison of relative expression of the clock genes Bmal1 in HNSCC cell line Cal-27. * p<0.05.

Comparison	P-value for acrophase difference	P-value for amplitude difference
Serum shock vs. aMT 10 nM	ns (0.614919)	<0.05 (0.037591)
Serum shock vs. aMT 100 µM	ns (0.198786)	ns (0.119811)
Control vs. aMT 10 nM	ns (0.657939)	ns (0.718146)
Control vs. aMT 100 µM	ns (0.908333)	ns (0.241201)
aMT 10 nM vs. aMT 100 µM	ns (0.6226)	ns (0.474727)
aMT 10 nM vs. aMT 500 µM	ns (0.783253)	ns (0.445071)
aMT 100 µM vs. aMT 500 µM	ns (0.299178)	ns (0.898303)

3. High doses of melatonin induce circadian expression of Sirtuin-1 in HNSCC cells.

Sirt1 plays a key role in the regulation of *Per2* circadian expression. In addition, in recent years, the NAMPT-NAD-SIRT1 axis and mitochondrial metabolism have aroused scientific interest. For these reasons, we studied the effect of melatonin on *Sirt1* expression (Table 8). The data demonstrate that 500 µM and 1000 µM doses of melatonin induced a synchronization of the *Sirt1* circadian rhythm as compared to the control group (Fig. 8).

Table 8. Cosinor analysis of relative expression of *Sirt1* in HNSCC cell lines Cal-27 and SCC9 after serum shock, control and aMT (500 and 1000 μM) treatments.

Cell line	Gene	Treatment	Presence of rhythmicity (p-value)	Acrophase	Amplitude
Cal-27	***Sirt1***	Serum shock	<0.05	8.240747	0.18581
		Control	ns	-	-
		aMT 500 μM	<0.01	10.27415	0.208889
		aMT 1000 μM	<0.01	12.19064	0.193523
SCC9	***Sirt1***	Serum shock	<0.01	7.629646	0.193267
		Control	ns	-	-
		aMT 500 μM	<0.05	11.1633	0.154587
		aMT 1000 μM	<0.05	9.559829	0.174447

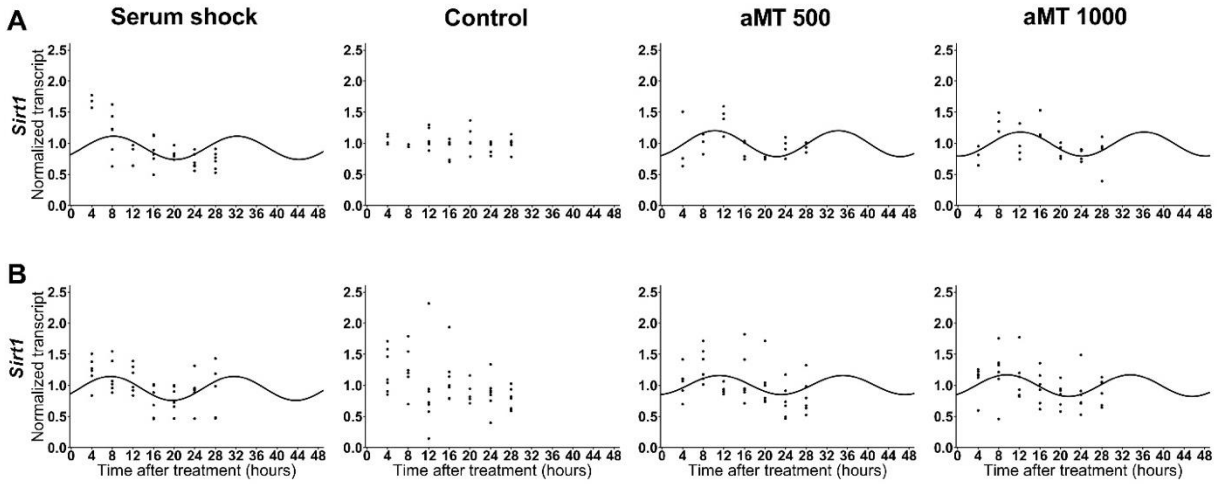

Figure 10. Relative expression of Sirt1 in HNSCC cell lines Cal-27 (A) and SCC9 (C) after serum shock, control and aMT (500 and 1000 μM) treatments**;** n= 3-6 independent experiments. The best cosinor fit is shown as a continuous line at a time of 48 h.

In addition, melatonin induced significantly advanced acrophase as compared to that obtained after serum shock (Table 9 anf Fig. 11). This confirms that melatonin at high doses directly affects *Sirt1* expression in HNSCC cells.

Table 9. Circadian rhythm comparison of relative expression of *Sirt1* in HNSCC cell lines Cal-27 and SCC9.

Cell line	Gene	Comparison	P-value for acrophase difference	P-value for amplitude difference
Cal-27	***Sirt1***	Serum shock vs. aMT 500 µM	ns (0.303475)	ns (0.832268)
		Serum shock vs. aMT 1000 µM	ns (0.073515)	ns (0.943527)
		aMT 500 µM vs. aMT 1000 µM	ns (0.271717)	ns (0.874045)
SCC9	***Sirt1***	Serum shock vs. aMT 500 µM	ns (0.087927)	ns (0.695747)
		Serum shock vs. aMT 1000 µM	ns (0.290038)	ns (0.842563)
		aMT 500 µM vs. aMT 1000 µM	ns (0.449046)	ns (0.844595)

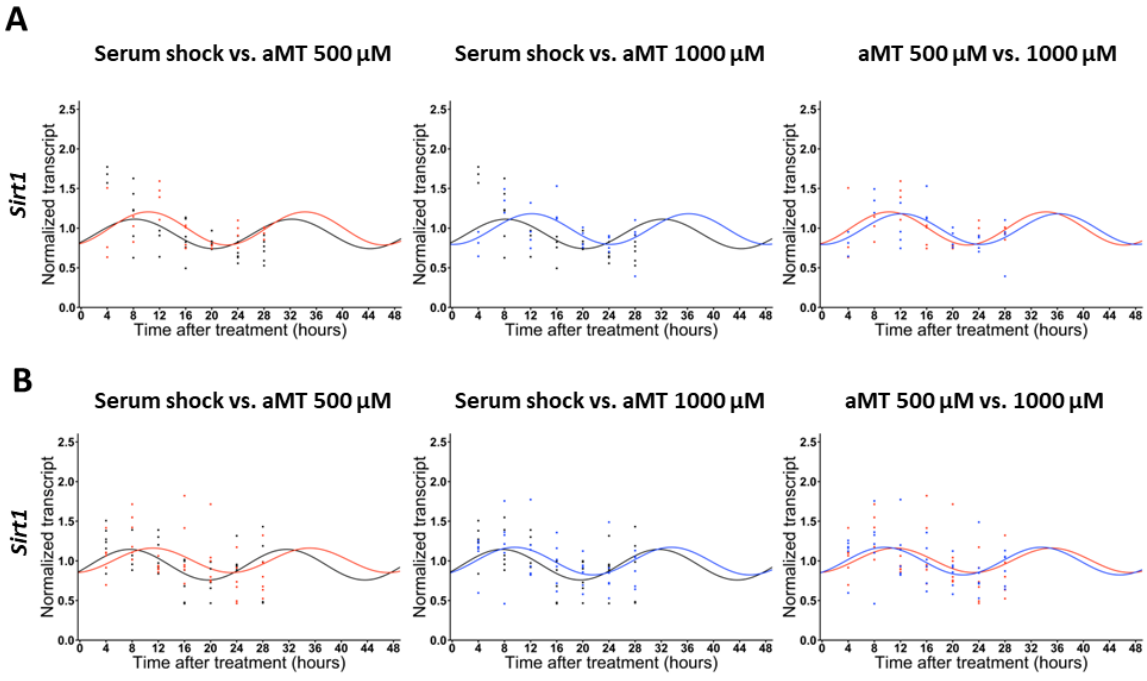

Figure 11. Cal-27 (A) and SCC9 (B) cosinor's best fits comparison of *Sirt1* between different experimental groups. Serum shock (black), control (green) and aMT 500 µM (red) and 1000 µM (blue) treatments; n= 3-6 independent experiments.

4. High doses of melatonin have no effect on the induction of a circadian rhythm of mitochondrial respiratory activity in Cal-27 cells

Next, we decided to determine whether the changes in the expression of *Bmal1*, *Per2,* as well as *Sirt1,* induced by melatonin, give rise to an alteration in mitochondrial respiratory activity. On the other hand, we also tried to determine whether an alteration in mitochondrial respiratory activity is responsible for an alteration in the expression of the genes mentioned. Thus, Cal-27 cells were subjected to the serum shock protocol in order to facilitate homogenous synchronization of clock gene expression. Table 10 shows the results of a systematic analysis. whereby cellular respiration in intact cells was assessed every three hours following synchronization. The OCR was measured, as described in section *2.5*. Figure 4 shows that Cal-27 cells, following synchronization, displayed a circadian rhythm of respiratory activity which peaked at h 17. However, we did not observe any changes in the rhythmicity of mitochondrial respiratory activity in either the control group or in the groups treated with high concentrations of melatonin (Fig. 12).

Table 10. Cosinor analysis of OCR in HNSCC cell line Cal-27 after serum shock, control and aMT (500 and 1000 μM) treatments.

Cell line	Treatment	Presence of rhythmicity (p-value)	Acrophase	Amplitude
Cal-27	Serum shock	<0.001	17.00138	0.311756
	Control	ns	-	-
	aMT 500 μM	ns	-	-
	aMT 1000 μM	ns	-	-
	Serum shock after control	<0.05	14.65534	0.143189
	Serum shock after aMT 1000 μM	<0.01	15.15964	0.225529

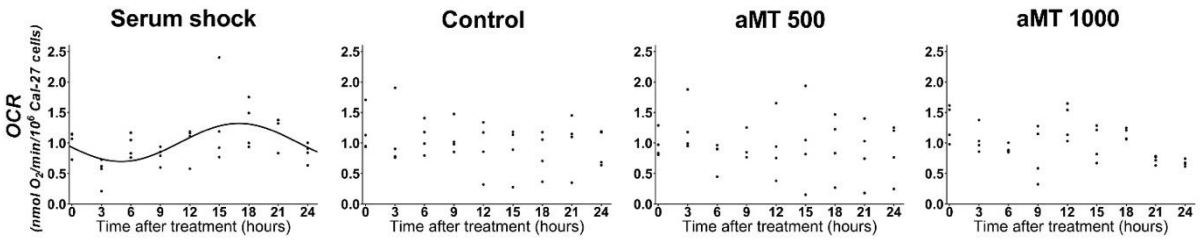

Figure 12. Measurement of mitochondrial respiratory activity in intact Cal-27 cells after serum shock, control and aMT (500 and 1000 µM) treatments. OCR refers to resting condition (*i.e.* endogenous substrate-sustained respiration); n= 3-6 independent experiments. The best cosinor fit is shown as a continuous line.

To rule out the possibility that mitochondrial respiratory activity was affected after high doses of melatonin treatment, we synchronized the cells following serum shock, as well as in control and after 1000 µM melatonin treatment (Figure 13). Table 11 and Figure 14 shows similar results for the group following serum shock. Respiratory activity showed a circadian rhythmicity. These data demonstrate that high doses of melatonin do not alter mitochondrial respiratory capacity.

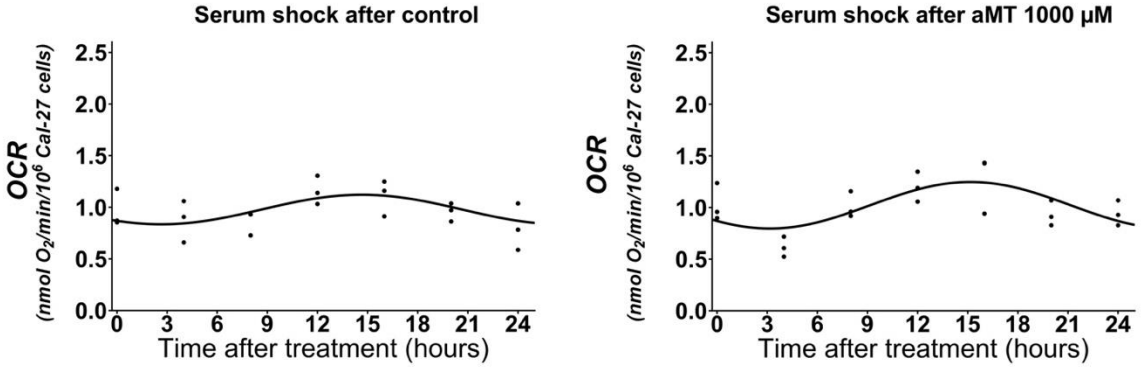

Figure 13. Measurement of mitochondrial respiratory activity in intact Cal-27 cells after serum shock, in control and aMT 1000 µM. OCR refers to resting condition (*i.e*. endogenous substrate-sustained respiration); n= 3 independent experiments. The best cosinor fit is shown as a continuous line.

Table 11. Circadian rhythm comparison of OCR in HNSCC cell line Cal-27.

Cell line	Comparison	P-value for acrophase difference	P-value for amplitude difference
Cal-27	Serum Shock vs. Serum shock after vehicle	ns (0,391999)	Ns (0,14612)
	Serum shock vs. Serum shock after 1000 µM aMT	ns (0,293366)	Ns (0,462536)

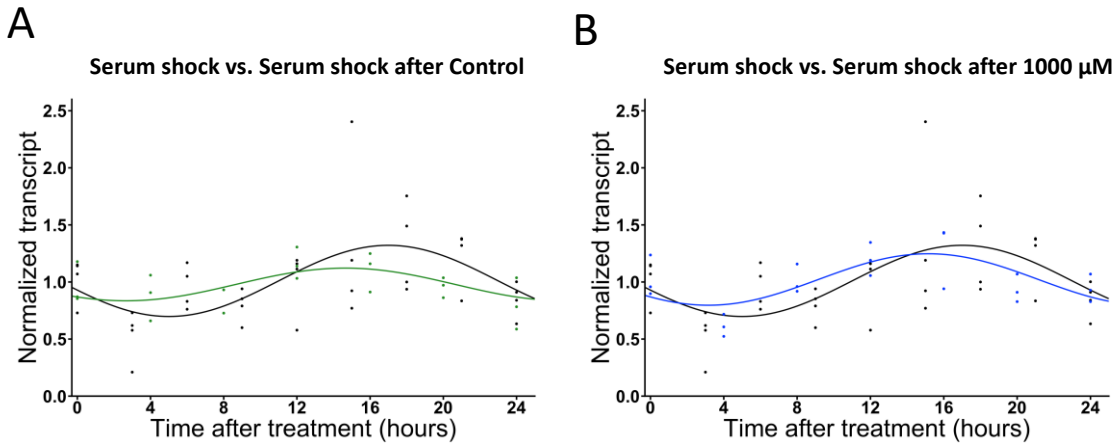

Figure 14. Cosinor's best fits comparison of OCR between different experimental groups. (A) Serum shock treatment (black) versus serum shock after control treatment (green); (B) serum shock treatment (black) versus serum shock after melatonin 1000 µM treatment (blue); n= 3-6 independent experiments.

5. Bmal1 knockdown increases cell proliferation while melatonin's antiproliferative effects are maintained

Given that Bmal1, a major link in mitochondrial activity, plays a key role in cell proliferation, we decided to study the antiproliferative effect of melatonin by reducing the expression of this clock gene. Figure 15 shows that, following the reduction in *Bmal1* expression by RNA interference, the proliferation of Cal-27 and SCC9 is significantly

increased. However, the antiproliferative effects of melatonin continued to be observed in a dose-response manner in both control and knock-down cells.

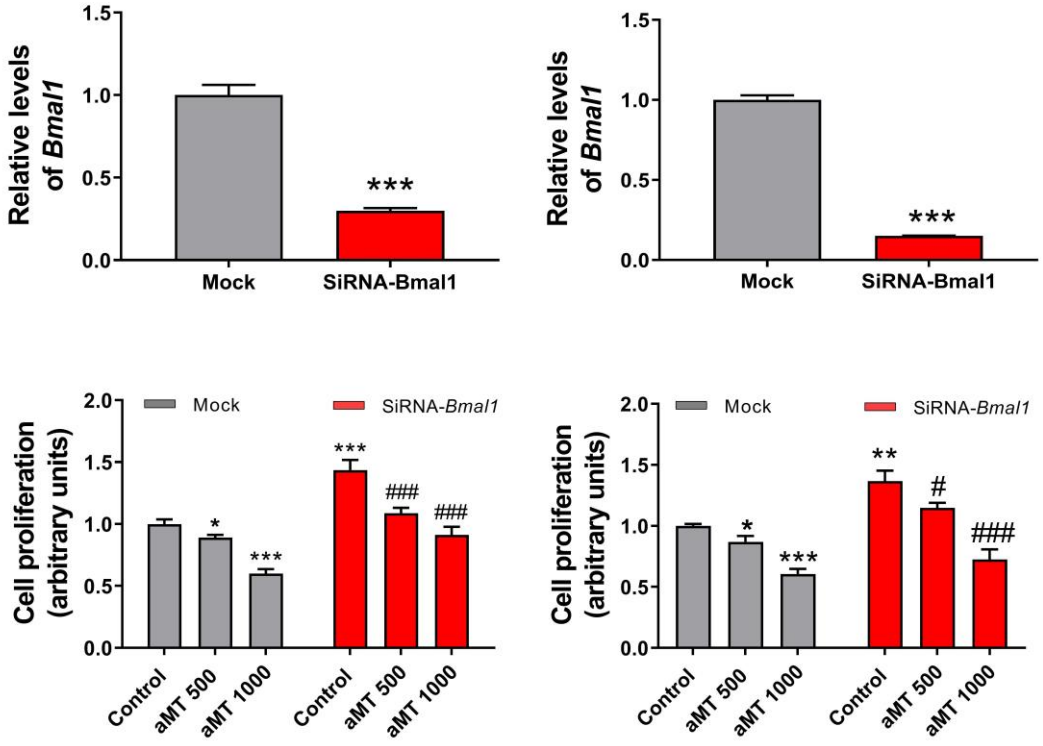

Figure 15. Effect of melatonin on Bmal1-silenced HNSCC cell lines Cal-27 (left) and SCC-9 (right). (A and B) Histogram showing *Bmal1* transcript level attained by q-RT-PCR in HNSCC cells transfected with siRNA-*Bmal1*. (C and D) effect of melatonin on cell proliferation assessed by CyQuant assay. Data are presented as mean ± SEM of n = 3 independent experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs*. mock control; # $p < 0.05$, ### $p < 0.001$ *vs*. siRNA-*Bmal1* control.

DISCUSSION

Previous studies have demonstrated that high concentrations of melatonin are required to exert its antiproleferative effects in HNSCC (Florido, Martinez-Ruiz, et al., 2022; Sung et al., 2020). However, the effects of high doses of melatonin on clock genes in these cancer cells has not been studied. Very few reports have highlighted the possible effects of melatonin on the circadian rhythm clock components in HNSCC cells (Jung-Hynes et al., 2010). The present study shows, for the first time, the results of high doses of melatonin on clock gene expression in HNSCC cells. We demonstrated that the antiproliferative effect of melatonin is not mediated by the clock gene *Bmal1*, and that melatonin treatment results in a resynchronization of the oscillatory circadian rhythm of the *Per2* and *Sirt1* genes. However, these changes in clock genes and in *Sirt1,* produced by melatonin, did not affect oscillations in mitochondrial respiratory activity. Having recently shown that the antiproliferative action of melatonin on HNSCC cells depends on its interaction with mitochondria (Florido, Martinez-Ruiz, et al., 2022), our results suggest that clock genes are not involved in the cytotoxic activity of melatonin in HNSCC cell lines, Cal-27 and SCC9. Therefore, this study has made it possible to explore how high doses of melatonin affect the cellular circadian machinery independently of its relationship with mitochondrial metabolism.

Currently, there is much scientific evidence what shows alterations in the circadian machinery in a variety of tumors, including head and neck cancer (Guo et al., 2020; Hsu et al., 2012b; Redondo et al., 2021). Moreover, alterations in *Bmal1* play a critical role in the regulation of cell proliferation in cancers, which are linked to an acceleration in tumor development and to modifications in responses to anti-cancer drugs (Hsu et al., 2012b; Tang et al., 2017). Moreover, the expression profiles of circadian rhythm components in cancers, as well as their alteration, depend on the

patient involved and/or the cell line. In this study, we demonstrated the presence of a circadian expression of *Bmal1* in Cal-27 and SCC9 cells. We also show that melatonin, at high concentrations, did not affect the resynchronization of *Bmal1*, although the rhythm of *Bmal1* was blunted at the melatonin dose of 1000 μM in Cal-27 with no effect on SCC9, possibly due to these cells being more sensitive to melatonin (Figure 7) (Florido, Martinez-Ruiz, et al., 2022; Guerra-Librero et al., 2021). Surprisingly, at levels closer to physiological doses (10 nM and 100 μM), melatonin does not resynchronize *Bmal1*, thus suggesting that low levels of melatonin have no effect on cancer clock genes in HNSCC cells. In addition, we recently demonstrated that high doses of melatonin significantly increased MT1 gene expression and also led to a marked reduction in MT2 and RORα expression (Guerra-Librero et al., 2021). These results reinforce the notion that high doses of melatonin do not disrupt receptor expression but may play a role, at least partly, in its impact in cancer cells. Our results therefore challenged the hypothesis that melatonin has an antiproliferative effect only at high doses.

To gain an insight into the antiproliferative effects of melatonin and given the importance of *Bmal1* in the regulation of the cell cycle and apoptosis (Matsumoto et al., 2016; Soták et al., 2014), we decided to inhibit this clock gene via RNA interference. Surprisingly, in both the knock down cell lines used in our experiments, the antiproliferative impact of melatonin was quite similar in both the presence and absence of *Bmal1*. This finding demonstrates that the antiproliferative effect of melatonin in HNSCC cells is independent of *Bmal1* in HNSCC cells.

Furthermore, alterations in Per2 have also been related to different types of cancers such as lung cancer (Papagiannakopoulos et al., 2016), breast cancer (Lesicka et al., 2018), and HNSCC (Hsu et al., 2012c). In this study, we demonstrate that *Per2*

expression in HNSCC does not exhibit a circadian rhythm (Fig. 6). However, the synchronization of Cal-27 and SCC9 cells induced a circadian expression in *Per2*. These findings enabled us to determine that circadian machinery is present in the two cell lines studied, which, however, lack regulated *Per2* clock gene expression. Interestingly, treatment with melatonin (1000 μM) altered *Per2* levels by resynchronizing the rhythmic gene expression pattern in HNSCC. These findings are consistent with Jung et al (2010) (Jung-Hynes et al., 2010), who showed that high concentrations of melatonin induce rhythmic *Per2* expression in prostate cancer cells. Per2 is an important tumor suppressor which regulates apoptosis by upregulating p53 and BAX and by downregulating c-Myc and Bcl-2 (Hua et al., 2006). These findings could facilitate the provision of additional data bolster the pro-apoptotic effect of melatonin in cancer cells.

An additional unexpected finding of this study is that mitochondrial respiratory activity does not fluctuate in a circadian manner following treatment with high concentrations of melatonin. This is of particular importance given the resynchronization of *Sirt1* expression observed after treatment with melatonin (Fig. 8). Numerous studies have addressed the role played by mitochondrial bioenergetics and dynamics in cell metabolism in relation to circadian rhythmicity, which is mainly due to PGC-1α, the target of Sirt1 (Bass & Takahashi, 2010; Coppi et al., 2021; Dhara et al., 2022). In addition, Cela et. al. (2016) (Cela et al., 2016) have shown that rhythmic respiratory activity is associated with NAMPT-NAD-Sirt1 axis and the oscillating acetylation/deacetylation state of complex I. However, we found that the circadian oscillations observed in *Sirt1* caused by melatonin does not translate into oscillations in mitochondrial respiratory activity, as was observed in serum shock-treated cells. In this regard, the serum shock protocol enables the clockwork machinery to be reset, which

facilitates the homogenous synchronization of cellular clock gene expression in culture once the repressing condition is released (Balsalobre et al., 1998). We can therefore conclude that melatonin exerts a specific effect on the expression of *Sirt1* which does not translate into mitochondrial changes.

In this study, we examined whether the antiproliferative activity of melatonin in HNSCC depends on clock genes. To this end, we explored the circadian machinery and its connection with mitochondrial respiratory activity. We were able to confirm that the antiproliferative effect of melatonin is independent of *Bmal1* and that melatonin is capable of resynchronizing *Per2* and *Sirt1* rhythmicity. However, these effects of melatonin on the circadian machinery do not alter the rhythmicity of mitochondrial respiratory activity. Overall, our findings suggest that the oncostatic effects of melatonin are exerted independently of the circadian clock genes and confirm previous data pointing to the involvement of a direct effect on the mitochondria. This study constitutes a novel approach to gaining a better understanding of melatonin's oncostatic mechanisms.

CONCLUSIONS

First: HNSCC cells lines Cal-27 and SCC9 lines present a circadian basal expression of *Bmal1*.

Second: high melatonin concentrations do not alter the circadian expression of Bmal1 in HNSCC, although the Bmal1 rhythm is attenuated by the 1000 μM melatonin dose in Cal-27 with no effect on SCC9, possibly due to an increased sensitivity of these cells to melatonin.

Third: Low doses of melatonin do not alter *Bmal1* expression, suggesting that low melatonin levels have no effect on cancer clock genes in HNSCC.

Fourth: The antiproliferative effect of melatonin was similar in both the presence and absence of *Bmal1* in HNSCC, suggesting that the antiproliferative effect of melatonin in HNSCC cells is independent of *Bmal1*.

Fifth: *Per2* expression does not exhibit a circadian rhythm in HNSCC.

Sixth: High concentrations of melatonin resynchronize the expression of Per2 in HNSCC suggesting that Per2 could have an important role to regulate apoptosis since Per 2 is an important tumor suppressor which regulates apoptosis by upregulating p53 and BAX and by downregulating c-Myc and Bcl-2.

Seventh: High concentrations of melatonin resynchronize the expression of *Sirt1* in HNSCC, while mitochondrial respiratory activity does not fluctuate in a circadian manner following treatment melatonin treatment.

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