THE MOLECULAR CLOCK IN THE SKIN, ITS FUNCTIONALITY, AND HOW IT IS DISRUPTED IN CUTANEOUS MELANOMA: A NEW PHARMACOLOGICAL TARGET?

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ABSTRACT

The skin is the interface between the organism and the external environment, acting as its first barrier. Thus, this organ is constantly challenged by physical stimuli such as UV and infrared radiation, visible light, and temperature as well as chemicals and pathogens. To counteract the deleterious effects of the above-mentioned stimuli, the skin has complex defense mechanisms such as: immune and neuroendocrine systems; shedding of epidermal squamous layers and apoptosis of damaged cells; DNA repair; and pigmentary system. Here we have reviewed the current knowledge regarding which stimuli affect the molecular clock of the skin, the consequences to skin-related biological processes and, based on such knowledge, we suggest some therapeutic targets. We also explored the recent advances regarding the molecular clock disruption in melanoma, its impact on the carcinogenic process, and its therapeutic value in melanoma treatment.

Key Words:

Skin Biology; Skin Physiology; Biological and Molecular Clock; ~~Circadian Clock~~; Skin Cancer; Carcinogenic Process ~~Melanoma~~.

THE SKIN

The skin is the largest organ in human body, and it accounts for 16% of total body weight. It consists of several specialized cells which make this organ sensitive to several stimuli such as touch, pain, pressure, itching, light and radiation, and temperature [1]. The epidermis is mainly comprised by keratinocytes, melanocytes, $CD8^+$ T, Langerhans and Merkel cells. It is divided into five layers: *stratum basale*, *stratum spinosum*, *stratum granulosum*, *stratum lucidum*, and *stratum corneum*. There are no blood vessels in the epidermis and, therefore, the dermis provides all the nutrients and oxygen to the epidermis [1,2].

The various layers of the epidermis represent different stages of keratinocyte maturation. In this process, the epidermal stem cells differentiate into keratinocytes which migrate upwards and begin to express several structural proteins, adhesion molecules, and lipid-producing enzymes. In the *strata lucidum* and *corneum*, keratinocytes lose their nuclei and form a lipid barrier – the epidermal barrier [1,3]. In addition to keratinocytes, in the *stratum basale* reside the melanocytes which synthesize the pigment melanin that protects the skin against the deleterious effects of ultraviolet radiation B (UVB), A (UVA), and visible light [4,5].

Below the epidermis is the dermis, which is mainly composed of fibroblasts and contains lymph vessels, nerve endings, hair follicles, and glands. The dermis also has several types of immune cells such as innate lymphoid, $CD4^+$ T and mast cells, macrophages, dendritic and invariant natural killer cells [2]. The dermis is divided into reticular and papillary layers: the latter contains the nerves and capillaries while the former is composed of collagen and elastic fibers, which provide the physical support [1,3].

The hypodermis is no longer considered as part of the skin [1]; however, its influence on the skin cannot be ignored. For instance, dermal adipocytes are involved in hair growth [6], wound healing [7], skin aging [8,9], temperature regulation [10,11], and immune response as well as influence the development and progression of several cutaneous disorders [12-14]. Additionally, visible light has been shown to affect *in vitro* mouse and human subcutaneous white adipocytes, leading to decreased lipid droplet size, increased basal lipolysis rate, and alterations in adiponectin and leptin secretion [15]. These findings, therefore, show an interesting and yet unexplored action of visible light on the hypodermis. In addition to its traditional histological classification, the skin can also be classified into 4 functional barriers: the microbiome barrier (skin surface), the

chemical barrier (*stratum corneum*), the physical barrier (epidermis), and the immune barrier (dermis) [2].

The skin is the first barrier that protects the organism [16,17] and thus it is susceptible to several external stimuli such as UVA, UVB and infrared radiation, visible light, temperature, chemicals, and pathogens [18,19]. To counteract such stressors, the skin displays elegant and complex mechanisms of defense such as: immune [3,20] and neuroendocrine systems [21,22], increased epidermal thickness and cytokeratin, regular shedding of epidermal squamous layers and apoptosis of damaged cells [23], DNA repair machinery [23-26], and skin pigmentary system that acts as a natural sunscreen [4,23,27-29]. Not surprisingly, many of these protective systems were shown to display rhythmic and circadian oscillation in their defensive role as described in depth below.

This review is divided into two sections. In the first section our focus will be to describe how external time is interpreted by the circadian machinery and how time affects the biological processes in the skin. In the second section, we will explore how the circadian mechanism is affected in cancer cells, focusing malignant melanocytes, the recent advances in the field, and putative therapeutic applications in melanoma treatment.

THE MOLECULAR CLOCK – HOW OUR ORGANISM KEEPS TRACK OF TIME

The statement: "Timing is everything" has an important meaning in many areas of science, and particularly in biology. The functions in the human body are not constant along the day. In fact, several biological functions have a time-dependent behavior such as: heart rate and blood pressure [30]; memory; cognitive performance; awareness and sleep onset [31,32]; DNA repair [24], and energy metabolism [33-35] among others. The temporalization and temporal segregation of physiological processes are prominent features that allow the organism to adapt to the oscillatory conditions posed by the environment. Since environmental alterations take place in a predictable fashion within 24 h period, the presence of a biological system able to track and interpret time, and based on this information, anticipate and regulate several physiological processes, is key for organism survival [36,37].

In mammals, the suprachiasmatic nuclei (SCN) are the central pacemaker [38], which is comprised of approximately ~~20,000~~ 16,000 neurons (8,000 in each nucleus) [39]. The retina is the responsible organ feeding the SCN with the external timing information: Light information is converted by a subset of intrinsically photosensitive cells that express

melanopsin (OPN4) – a photopigment – into electric stimuli that reach the SCN through the retinohypothalamic tract [40-42]. At the core of this temporal controlling mechanism lie the clock genes, whose oscillation along the day is the molecular basis for keeping track of the time. The clock gene machinery may be explained in the following way (Figure 1): *Clock* (Circadian Locomotor Output Cycles Kaput) (or *Npas2*, Neuronal PAS-containing protein-1, in the SCN) and *Bmal1* (Aryl hydrocarbon receptor nuclear translocator-like protein 1) are translated into proteins in the cytoplasm and form the heterodimer CLOCK:BMAL1, which migrates to the nucleus where it binds to genes harboring E-box sequences in their promoters. Among these genes, *Per* (Period) and *Cry* (Cryptochrome) are transcribed and once translated into proteins they form heterodimers PER:CRY [43-45] that are phosphorylated by casein kinase 1δ (CK1δ) or CK1ε and migrate back to the nucleus [46,47]. The heterodimer PER:CRY inhibits the transcriptional activity displayed by CLOCK:BMAL1 [47,48]; in addition to clock genes, several other genes also harbor E-box elements in the promoter regions, rendering these genes clock-controlled (CCGs, Figure 1) [49].

Another loop of regulation is also exerted through E-box activation: CLOCK:BMAL1 activates *Rev-Erbα/β* (also known as *Nr1d1/2*) and *Rorα/β* (also known as *Nr1f1/2*) expression. REV-ERBα/β competes with RORα/β for the orphan receptor response element (RORE) sequence present in *Bmal1* promoter. It is known that REV-ERBα/β inhibits while RORα/β stimulates *Bmal1* expression [50-52]. Since PER:CRY inhibits the stimulatory action of CLOCK:BMAL1, consequently less *Per* and *Cry* genes are transcribed, and in addition to the continuous degradation of PER and CRY proteins, the inhibition displayed by PER:CRY on CLOCK:BMAL1 is reduced, and then CLOCK:BMAL1 binds to E-box regions and a new cycle of transcription starts [46,49,53-57]. Importantly, when light information reaches the SCN, a glutamate signaling takes places that leads to the fast transcription of several genes such as *Per* [58,59] with subsequent clock gene machinery reset (Figure 1).

The clock gene machinery is not restricted solely to the SCN. In fact, to date the clock gene machinery has been found in nearly all body cells, which shows that each tissue has its own local temporal control [49,53]. Approximately 5 to 20% of genes expressed in several organs were demonstrated to undergo circadian oscillation at the mRNA level [60]. Based on the temporal information from the environment, the SCN signals to several regions in the brain which control many physiological processes through nervous outputs, hormones, and body temperature [53,61]. Light and temperature

information from the environment is considered first order *zeitgebers* (time giver or synchronizer), but only light feeds the SCN with the external time, as SCN neurons are irresponsive to temperature variation. The organism also uses second order *zeitgebers* such as activity, food intake, body temperature, and social interaction to reset the various peripheral clocks [37,62]. These z*eitgebers* may be redundant and allow the whole organism to be under the influence of a single time zone, i.e., in internal synchrony.

Interestingly, this internal synchrony between the central and peripheral pacemakers is viewed as one of the – if not the most – important role of the circadian system [37,57,62]. Not surprisingly, this internal synchrony is acutely lost in people with jetlag syndrome or is chronically disrupted in shift workers [63,64], and the loss of proper timing, i.e. chronodisruption, is associated with several diseases such as neurodegenerative, metabolic and sleep disorders, and cancer [37,63-68].

CIRCADIAN ORGANIZATION OF THE SKIN

How clock genes are regulated within the skin and how the SCN controls the skin clock is still not fully comprehended. Thus far, only one study has addressed this question [69]. As expected, the mouse skin displays rhythmic expression of clock genes when the animal is kept in light-dark cycle (LD) or in constant dark (DD). Upon SCN lesion, the oscillatory profile of clock gene expression in the skin is lost. Thus, based on this study [69], it has been suggested that the clock gene machinery within the skin is "blind" to external stimuli, being only regulated by the SCN [70] (Figure 2).

Accordingly, the skin clock fits the so-called "Orchestra Model" [71,72]: In this model, the central clock displays a coordinating role among different organs and systems, i.e., each organ "plays its own instrument" but the coordination and synchrony of the song is exerted by the central clock. However, recently, a new model of organization between the central and peripheral clocks has been set forth: The "Federated Model", which is based on animals with specific deletion of *Bmal1* in the SCN [62,73] or in the forebrain [74] rather than physical lesion of the SCN. When mice were subject to light-dark (LD) cycles, a rhythm of locomotor activity was found, but in DD no rhythm was detected [73-75]. A similar profile was found for clock gene expression in peripheral organs, i.e., when mice were kept in LD the expression of peripheral clock genes was synchronized and the phase was preserved while in DD clock gene rhythm still persisted, although dampened [73].

The new insights and knowledge brought by the "Federated Model" have opened a new perspective regarding the regulation of peripheral clocks exerted by the SCN. In this line, the "Federated Model" suggests that the SCN is required to synchronize the organism only in the absence of *zeitgebers*; in this scenario the peripheral clocks rely on coordinating timing signals from the SCN, resembling a hierarchical organization. When *zeitgebers* are present, peripheral clocks are aligned to external timing, in a "federated" and SCN-independent fashion [62].

Unfortunately, how the molecular clock of the skin behaves when *Bmal1* is selectively knockout in the SCN has not been investigated. Nonetheless, an interesting study has shown that restricting feeding time affects the phase and amplitude of several diurnally expressed clock and clock-controlled genes [76]. These data have unexpectedly shown that a classic metabolic *zeitgeber* also modulates the molecular clock of the skin [76]. Further studies are required to understand the role of the SCN and other *zeitgebers* in the skin clock.

Skin Molecular Clock: A Historical View

The first evidence of clock genes in the skin was provided in 2000 by Zanello and colleagues. The authors demonstrated the presence of *CLOCK* and *PER1* mRNA and protein in primary cultures of human keratinocytes, melanocytes, dermal fibroblasts, and in A375, an immortalized melanoma cell line [77]. In the following year, the expression of *CLOCK*, *PER1*, *CRY1*, and *BMAL1* was detected in human skin and oral mucosa. *PER1*, *CRY1*, and *BMAL1* displayed rhythmic expression while *CLOCK* was constitutive [78]. In 2002, a study demonstrated that the molecular clock of primary culture of human keratinocytes was responsive to ultraviolet radiation B (UVB, 0.1 kJ/m^2) since this stimulus led to a downregulation of *PER1*, *CLOCK*, and *BMAL1* expression [79].

From 2002 to 2011, no investigation – to the best of our knowledge – addressing the role of clock genes in the skin was published, and only in 2012 the interest in the molecular clock of skin cells reemerged. Currently, it is widely accepted that fibroblasts, keratinocytes, and melanocytes have a local circadian machinery, which displays phase relationship and period of clock gene expression in a cell specific manner after dexamethasone synchronization [80]. These data, therefore, argue in favor of a local multi-oscillatory temporal system in skin that could be responsible for controlling several aspects of this organ in a time-dependent fashion [70,80,81]. It is known that the functionality of the biological clock in the skin starts two months after birth while in aged

rats the phase of clock gene expression is more variable compared to younger animals [81].

Recently, an elegant study demonstrated that the reconstruction of 24 h long gene expression was possible by sampling a small number (n = 20) of individuals along 24 h and a larger population at a single time point (n = 219). With this approach, hundreds of rhythmically expressed genes were found in human epidermis; in addition, several genes were classified as biomarkers, and thus they could be used to easily assess the circadian time of patients [82].

Over the past decade excellent reviews addressing the role of circadian clock in the skin have been published [3,26,70,83-86]. In the next pages, we will address the effects of ambient and endogenous factors on the skin biological clock (Figure 2), and the processes known to be regulated by the molecular clock of the skin (Figure 3). We also invite the reader to explore insightful ideas and views about the cutaneous circadian clock [87].

Ambient and Endogenous Stimuli Affecting the Skin Clock

UV radiation and visible light

The skin possesses a photosensitive system, comprised by opsins, that can detect light and trigger biological events in response to light stimulus [88-97]. In addition to the opsin-mediated light perception, the skin also senses light and radiation through their interaction with chromophores other than retinaldehydes such as flavins, porphyrins, nitrosated proteins (as reviewed by Garza and colleagues [98], and melanin [99].

In synchronized human keratinocytes, UVB radiation (280 – 320 nm, 0.2 kJ/m^2) was shown to immediately downregulate the expression of *CLOCK*, which was normalized 12 h later [100]. Similar phenomenon has been previously appreciated by Kuwara and coworkers [79]. In the skin of human volunteers, 6.88 kJ/m^2 of UV radiation (64% UVB, 16% UVA, and 19% visible light) also led to a downregulation of *CRY2* 24 h after irradiation compared to non-exposed controls [101]. Based on the literature, we suggest that in both scenarios, the mechanism underlying UVB-induced clock gene downregulation may be a UVB-induced ATP reduction, an event known to lead to a phenomenon called UVB-induced energy crisis [102,103]. In addition to the above-mentioned studies, UVB radiation (0.05 kJ/m^2) was shown to acutely reduce *BMAL1* and *CLOCK* transcripts, but 24 h later *BMAL1* and *CLOCK* levels were increased and normalized, respectively, in immortalized and primary culture of human keratinocytes

[104]. Upon *BMAL1* or *CLOCK* gene silencing, increased cell viability and reduced number of late apoptotic cells were found. The reduced UVB-induced apoptosis was correlated with decreased expression of the cell cycle inhibitor p21, and the DNA damage marker γ- H2AX, in immortalized keratinocytes and in terminally differentiated primary keratinocytes [104]. Furthermore, Park and coworkers [100] found reduced mRNA levels of *Tissue Inhibitor* of *Metalloproteinase* 3 gene (*TIMP3*) due to UVB-induced inhibition of mRNA synthesis, or when *CLOCK* was silenced. Remarkably, upon UVB radiation the reduction on *CLOCK* and *TIMP3* transcripts resulted in increased secretion of metalloproteinases (MMP), TNF-α, and inflammatory cytokines, events that contribute to the well-known effects of UVB-induced inflammation [49]. Another link between the molecular clock and MMP was recently reported as knockdown of *PER2* and *PER3* increased the expression of MMP1 mRNA and protein in immortalized human keratinocytes, in which PER3 reduced the expression of MMP1 in a cAMP-dependent pathway [105]. Remarkably, *PER1* was shown to act as positive regulator of MMP1.

In human skin, UVB radiation (1 to 4-fold the minimal erythema dose) led to increased erythema index in the same skin phototype II and III individuals who received the radiation in the evening compared to the morning. Interestingly, basal CRY2 expression correlated with erythema response, and increased p53 protein was reported in morning exposed-subjects compared to evening-exposed ones [101]. Additionally, UV radiation (6.88 kJ/m^2, 64% UVB, 16% UVA, and 19% visible light) downregulated *CRY2* in human skin but upregulated *CRY1* expression in subcutaneous adipose tissue, which shows an interesting response to UVB radiation in a tissue that is not directly affected by UVB radiation [106].

Recently, UVC (0.05 kJ/m^2) radiation has been shown to synchronize the molecular clock of NIH-3T3 fibroblasts; however, the cytotoxic effect of UVC was not investigated and, therefore, the physiological relevance of such data is difficult to assess [107]. In addition, NIH-3T3 embryonic fibroblasts harboring *Per2* promoter-driven firefly luciferase (*Per2-Luc*), upon genotoxicity dose of UVB radiation (254 nm, 20 to 200 kJ/m^2), showed a rhythmic *Per2* expression pattern. The UVB-induced *Per2* expression was absent in embryonic fibroblasts of *Heat shock factor* (*Hsf*) knockout mice. Chromatin immunoprecipitation assays (ChIP) demonstrated that UVB radiation induced HSF1 binding to the heat shock response element (HSE) site 2 on *Per2* promoter, thereby enhancing its expression. Interestingly, p53 can suppress *Per2* expression hours after UVB radiation, thus modulating its expression. It was shown that UVB radiation firstly

activated HSF1 pathway, which then activated p53, and that BMAL1 regulated both pathways through BMAL1/HSF1, leading to molecular clock synchronization [108].

Most of the studies in the literature are focused on keratinocytes or fibroblasts, and only a few on melanocytes. Our group has shown that visible light (400 – 700 nm, 0.85 kJ/m^2) and UVA radiation (365 nm, 4.4 kJ/ m^2) have a minor effect on the molecular clock of murine melanocytes when compared to malignant cells [93,109]. We have found a correlation between the increased levels of melanin content and reduced clock expression in malignant cells compared to normal cells [93,109,110], a phenomenon initially demonstrated by Hardman and colleagues [111] in clock gene-silenced human melanocytes.

Temperature

In addition to detecting light, the skin is also equipped to detect temperature via classically known thermoreceptors such as the temperature-sensitive transient receptor potential (TRP) channels [112-115]. Our group has demonstrated that normal and malignant melanocytes sense temperature (39ºC for one hour) that leads to biological clock activation in a non-canonical melanopsin-dependent fashion [116]. Thus, our findings in melanocytes along with the data in human sperm cells [117], clearly demonstrate that opsins are dual sensors, detecting both light and temperature in mammals (Figure 2) [118], a concept that has been previously established in *Drosophila* larvae [119-121].

Human skin displays an oscillatory profile on surface temperature with higher and lower values in the evening and morning, respectively [122,123]. In 2011, Sporl and colleagues showed that temperature cycles (37/33ºC during 12/12 h) entrained the biological clock of dexamethasone-synchronized human keratinocytes, in which several rhythmic genes were identified. Within this line, approximately 300 genes have been shown to oscillate in a circadian manner in human epidermis; among them, *Krüppel-like factor 9* (*KLF9*), which is involved in proliferation and differentiation of keratinocytes, was identified as a responsive gene to cortisol and temperature cycle [124].

The first link between clock gene activation and temperature was reported by Tsuchiya and colleagues in mouse NIH-3T3 fibroblasts [125]. Several years later Tamaru and coworkers showed that *Per2* expression was induced by heat shock (43°C) in NIH-3T3 mouse fibroblasts, and upon knockout or mutation in *Hsf* the heat-induced clock activation was lost [126]. In primary culture of rat dermal fibroblasts kept in temperature

ranging from 32 to 39ºC, an oscillation on *Per1* expression was found; however, cells kept at 32 and 35ºC showed increased *Per1* gene amplitude compared to the ones maintained at 37 and 39ºC [81]. There is just one report in the literature regarding the effect of temperature on the molecular clock of melanocytes: Our group demonstrated that heat pulse (39.5ºC during 1 h) increased *Per1* transcript levels in synchronized murine melanocytes [116].

In vivo experiments are also scarce. In skin of hairless rats kept under DD or LD cycles at constant temperature (22°C), trans-epidermal water loss (TEWL) and *stratum corneum* hydration (SCH) displayed an oscillatory pattern; however, LD cycles with high temperature (28°C) in the light phase affected the TEWL but not the SCH rhythms [127]. It is a well-known fact that the skin temperature in humans is lower than core temperature [128,129] and that it is not constant, i.e., it oscillates displaying its lowest levels at night compared to the day in kids and young adults of both sexes [123,130]. Although core temperature is classically assessed by a rectal thermometer, which is the gold standard method, evaluation of skin temperature has been suggested as an alternative method [131,132] to evaluate circadian rhythms [133,134] and a putative marker for circadian disruption in humans [133,135-137].

Reactive oxygen species

The skin possesses several complex protective mechanisms against oxidative stress that assure its homeostasis against external threatening agents such as UV radiation, visible light, temperature, chemicals, as well as endogenous reactive oxygen species (ROS) [3,18,138]. Components of the antioxidative system include catalase (CAT) [139,140], glutathione peroxidase (GPx) [141,142], superoxide dismutase (SOD) [143,144], which are known to be rhythmically active, and antioxidants like vitamins A, C, and E, melatonin, and glutathione (GSH) [3,138,145]. In the literature few studies relate the molecular clock with oxidative stress in the skin [138]; on the other hand, the link between molecular clock and oxidative stress in other organs, for instance the liver, is well comprehended [146,147]. Interestingly, it has been shown that the epidermis displays a higher antioxidant activity compared to the dermis [148].

The molecular clock of keratinocytes has also been linked to antioxidant responses since synchronized human keratinocytes (HACAT cells) exhibited a more efficient response, mainly by a faster migration of the nuclear factor erythroid 2–related factor 2 (NRF2) protein to the nucleus, and rapid induction of *Bmal1* transcription compared to

non-synchronized cells [149]. In another line of human epidermal keratinocytes (NHEK), a decrease in ATP synthesis and an increase in hydrogen peroxide (H_2O_2) level were found in synchronized cells after nutritional starvation, or UVB (0.1 kJ/m^2) irradiation, compared to unsynchronized cells [150]. One has to bear in mind, however, that the above studies [149,150] compared two different cell populations (synchronized vs. non-synchronized), and the diverse results may be due to factors other than a direct link with the molecular clock. On the other hand, a strong evidence linking anti-oxidative activity and circadian system was provided ~~In~~ in mouse NIH-3T3 fibroblasts, in which near-lethal doses of ROS ~~were reported to~~ reset the ~~biological~~ clock in a *Bmal1*- and *Hsf*-dependent fashion, leading to cell survival [151].

Hormones

The skin is widely recognized as a neuroendocrine organ that displays equivalents to the hypothalamic–pituitary–adrenal (HPA) and hypothalamic–pituitary–thyroid (HPT) axes, catecholaminergic, cholinergic, steroidogenic, melatoninergic and secosteroidogenic systems, that regulate several cutaneous biological processes [22,152-156].

Melatonin is an indoleamine synthesized during the night by the pineal gland, known as one of the most important hormones involved with circadian synchronization of peripheral tissues. Melatonin translates the photoperiod information into a hormone signal [157]. It is also synthesized in a variety of extrapineal organs [158], including the skin where melatonin is produced locally under both physiological and pathological conditions and is an important player in skin biology [159,160]. Classically melatonin synthesis involves the participation of arylalkylamine N-acetyltransferase (AANAT), which catalyzes the conversion of serotonin into N-acetylserotonin (NAS), and acetylserotonin O-methyltransferase (ASMT), which catalyzes the methylation of NAS into melatonin [159,161-165]. AANAT is the rate-limited enzyme in the pineal gland being activated by nocturnal noradrenalin stimulation. Mammalian skin possesses the entire enzymatic machinery to synthesize melatonin, including AANAT. Interestingly, C57 mouse strain does not express functional AANAT [166], but are proficient in skin melatonin production. In this condition, melatonin synthesis is allowed by the action of arylamine N-acetyltransferase (NAT), that leads to serotonin acetylation in an AANAT-independent pathway [161,167].

Interestingly, melatonin levels are higher in human epidermis compared to circulating levels, which argues in favor that skin-produced melatonin is mainly used locally in the skin; moreover, melatonin levels in the skin is dependent on race, gender, and age, and African-American skin shows the highest level of this hormone compared to Caucasian skin [160,163]. An interesting difference between human and mouse skin lies on the fact that the former expresses melatonin receptor 1 (MT1) and (MT2) [168,169] while mouse skin predominantly expresses MT2 [170,171], which are both affected by UVB radiation [169]. Regarding its metabolism, melatonin can be degraded in the skin not only via indolic or kynuric pathways [163,172,173], but also by a direct and non-enzymatic action of UVB radiation [174].

This indole hormone displays several protective functions in the skin such as, but not limited to, protection against light and oxidative stress, induction of DNA repair, regulation of barrier function, pigmentation, and immune system, and thermoregulation (reviewed in [159,163]. In fact, the melatoninergic system of the skin protects against UV radiation [175,176], through an interaction with mitochondrion function [159,162], and by increasing the levels of several antioxidant enzymes [177], consequently leading to the attenuation of UV-induced DNA damage [177,178].

Therefore, melatonin could be pharmacologically exploited to treat skin related diseases, cancer, and aging [161,176]. Despite its well-known effects on skin biology, only an *in vitro* study has evaluated the effect of this hormone on the molecular clock of skin cells in which melatonin applied, at different times of the day, affected the molecular clock of rat primary culture of dermal fibroblasts [81]. In addition, in a non-mammalian vertebrate, it was demonstrated that *Per1* displayed a circadian oscillation when *Xenopus laevis* melanophores were submitted to LD cycles [179]; melatonin applied in the photophase, but not in the scotophase, led to a reduction of *Opn4x* transcripts [180], the opsin responsible for light perception in this model [181,182], which could lead to impairment of the circadian regulatory system in melanophores.

The modulation of the molecular clock of human hair follicle by thyroxine (T4), a hormone from the HPT axis, has also been described. Twenty-four hour long T4 treatment ~~however, the role of the biological clock controlling the neuroendocrine system of the skin remains elusive. In human hair follicles, 24 h treatment with thyroxine (T4)~~ led to a reduction on mRNA and protein levels of BMAL1 and PER1. In long-term treatment (> 6 days) mRNA level of several core clock genes were upregulated compared

to control, whereas PER1 and BMAL1 showed increased and unchanged protein expression levels, respectively [183].

Glucocorticoids are classically associated with the regulation of the molecular clock *in vitro* and *in vivo* [184,185]. In fact, glucocorticoids, as well as melatonin, are important hormonal temporal cues for the organism [37,53]. The skin possesses its own local steroidogenic system that is closely regulated [186-189]. Interestingly, UVB radiation has been shown to affect the central and the skin HPA axes [190-192]. However, just one study has evaluated the action of dexamethasone on the skin molecular clock: dexamethasone synchronizes the molecular clock of primary culture of human fibroblasts, keratinocytes, and melanocytes, which showed cell-type-specific oscillating periods [80]. These findings first demonstrated the concept of several molecular clocks in the skin [80]. Estradiol, another hormone synthesized by the skin [22,193,194], was shown to activate the molecular clock of malignant murine melanocytes while no effect was found in normal cells, which was associated with a reduction on melanin content only in malignant melanocytes [110].

Although the skin displays an important part in the biosynthesis of vitamin D [22,195], only one study demonstrated the role of vitamin D in the synchronization of the molecular clock of adipose-derived stem cells [196]. Interestingly, it has been shown that endogenous non-calcemic and non-classical vitamin D derivatives may act as antagonist and/or inverse agonist of RORα/γ [197,198], and that vitamin D protects against UVB-induced DNA damage via activation of DNA repair pathways which are dependent on NRF2 and p53 [199].

Food

Restricted food regimens are known to affect the molecular clock of metabolic organs [200], but their effect on skin clock was unknown, although they modify several traits in skin physiology and pathology [201,202]. Based on this gap of knowledge, Wang and coworkers (2017) demonstrated that time-restricted feeding shifted the phase and altered the amplitude of many diurnally expressed clock and clock-controlled genes in the skin. It also affected the expression of about 10% of skin transcriptome, most prominently metabolic genes; nevertheless, restricted feeding regimens did not alter the phase of DNA synthesis. Remarkably, mice fed at night showed increased sensitivity when exposed to UVB radiation during the night than during the day [76].

pH

The oscillation of clock gene expression in primary culture of human skin fibroblasts kept in medium with pH 6.7 displayed shorter period, increased amplitude, and slower damping rate compared to cells kept in pH 7.2. These effects were not related to cellular death and/or viability, thus demonstrating a direct effect of pH on the biological clock of fibroblasts [203]. These data show how important is to rigorously control medium pH in assessing temporal variation of gene expression.

Biological Processes Regulated by the Molecular Clock of the Skin

Pigmentation

Another prominent feature of the skin is its ability to produce melanin, a polymer that blocks and mitigates the UV-induced damage [204-206]. In 2015, Hardman and colleagues first reported that the molecular clock negatively controls the pigmentation process, through gene silencing strategies, in human melanocytes [111]. In the following year, our lab corroborated these findings as we demonstrated a reduction on the molecular clock expression with increased levels of melanin content in malignant melanocytes compared to healthy cells [93,110]. Interestingly, we reported that estradiol did not affect melanin content in normal melanocytes but reduced pigmentation in malignant cells. This effect was associated with an increase on clock gene expression shown by malignant melanocytes while no effect was seen in normal cells [110].

Immune system

One of the first reports about skin rhythmic response was made by Reinberg and colleagues who demonstrated a circadian profile in erythema and weal in response to histamine injections in human subjects [207]. The skin immunity is dependent on conserved pattern recognition receptors (PRRs), including several isoforms of toll-like receptors which play an important role in innate immune system. This system provides the response to a variety of immunogenic stimuli including bacterial, fungal, and viral molecules [20,208]. In addition, the skin also harbors an immune adaptive system that leads to humoral and cell-mediated immunity [20].

In *Clock* or *Per2* knockout mice imiquimod-induced dermatitis improved and worsened, respectively, compared to wild type mice. These responses were associated with decrease or increase of *interleukin 23 receptor* (*IL-23R*) in γ/δ+ T cells in *Clock* or *Per2* mutant mice, respectively [209]. A time-dependent reaction in passive cutaneous

anaphylactic reaction is lost in *Per2* knockout mice, which also display reduced sensitivity of mast cells to the inhibitory effects of glucocorticoid both *in vitro* and *in vivo* [210]. Remarkably, increased incidence of psoriasis has been reported in rotating night shift women [211].

In atopic dermatitis, *RORα* has been shown to restrain allergic skin inflammation. Deletion of *RORα* did not affect the number of skin-resident T regulatory cells (T_{regs}) but increased the type 2 allergic skin inflammation in response to a topical agent. In fact, the deletion of *RORα* led to increased inflammatory influx that included T cells, basophils, neutrophils, mast cells and a selective enrichment in eosinophils, and altered the expression of important genes involved in chemotaxis, function and inflammation of T_{regs} [212]. The role of the molecular clock in atopic dermatitis has been recently reviewed [213]. Interestingly, nocturnal pruritus has been associated with disruption in circadian rhythms, but its underlying mechanism is still poorly known [214]. For more in-depth understanding of the role of the biological clock in the skin immune system, the reader is referred to other reviews [3,70].

DNA repair

The skin is chronically exposed to UV radiation and temperature, which are known factors that can generate DNA damage [55,205,206,215,216]. DNA repair is, therefore, an important feature among the skin defense mechanisms. Interestingly, DNA damage in response to ionizing radiation has been reported to reset the molecular clock *in vitro* and *in vivo* [217]. DNA repair has been shown to oscillate in murine skin with increased activity in the afternoon/evening and decreased activity in the morning [24]. The relevance of these findings is clear when mice exposed to UV radiation in the morning displayed increased number of skin tumors when compared to mice exposed in the afternoon/evening [24]. Since DNA repair is less efficient in the morning, the UVB-induced damage is associated with DNA polymerase stalling in regions harboring unrepaired DNA damage, which leads to increased p53 activity with subsequent apoptosis and erythema [25].

In quiescent and dexamethasone-synchronized human fibroblasts, DNA damage induction and repair efficiency in response to UVB radiation are time-dependent [218]. In human skin, CRY2 basal expression correlated with erythema response, and increased p53 protein expression was reported in morning UVB-exposed individuals compared to evening-exposed ones [101]. Moreover, in lymphocytes of healthy volunteers daily

oscillation and higher activity in the morning of 8-oxoguanine DNA glycosylase (*OGG1*) level compared to the evening were reported. As expected, pharmacologically-induced oxidative DNA damage in lymphocytes was lower in the morning than in the evening. Remarkably, the molecular clock was associated with OGG1 oscillatory profile since *BMAL1* knockdown in human fibroblasts abolished *OGG1* circadian oscillation but increased OGG1 activity, thus leading to a faster oxidative injury repair [219]. A very interesting review on the effects of circadian clock on DNA repair has been published [26].

Cellular division and stem cell growth

Another important aspect of the skin is the different gene expression profile found in skin at telogen and anagen phases since the former has a predominant set of genes involved in cell division, circadian rhythm and metabolism [220]. Furthermore, in telogen skin there is a temporal segregation between the oxidative phosphorylation, known to produce increased ROS, and S-phase – the most susceptible stage of DNA. Remarkably, when *Bmal1* is specifically deleted in keratinocytes, time-dependent cell division is obliterated, leading to constitutively elevated cell proliferation and ROS levels [220].

In the epidermis, a daily self-renewal process takes place, in which epidermal stem cells differentiate into keratinocytes that undergo proliferation followed by terminal differentiation [221,222]. The skin, hair follicles, and sebaceous glands are known to possess a population of stem cells [70,222,223].

In mice, epidermal progenitors display the highest proportion of S-phase during the night (3 AM) while in humans it peaks in the afternoon (3 PM) [224]. It is worth to note that S phase takes places when glycolytic activity and oxidative phosphorylation are at its highest and lowest, respectively; thus, avoiding the elevated levels of ROS when DNA is at its most susceptible stage [225]. Mouse epidermal stem cells showed a similar pattern and displayed higher proliferation rate at night compared to day [226]. Interestingly, upon specific deletion of *Bmal1* in keratinocytes, the proportion of cells in *S* phase was high and constant, which clearly shows the important role of *Bmal1* in regulating cell cycle and proliferation in a time-dependent manner [220]. Within this line, *Klf9* was classified as a circadian transcriptional factor that control cell proliferation in the epidermis [124].

It is interesting to observe that human epidermis displays the S phase in the afternoon, when solar radiation is at its highest; thus, making our skin particularly

sensitive to UV and visible light deleterious effects. It is yet to be defined whether this clock-regulated mechanism contributes to skin cancer incidence in humans [24]. For a more detailed aspect regarding the role of clock genes in stem cells, the reader is referred to two reviews [70,222].

Hair follicle growth

Hair growth cycle is a remarkable clock-regulated process [227,228]. The hair cycle starts with an involution process in which most of the epithelial cells undergo apoptosis, the so-called catagen, followed by telogen, in which the hair follicle remains quiescent. Anagen is the stage where the activation of epithelial stem and progenitor cells, localized in bulge and secondary hair germ, and the proliferation and differentiation of progeny keratinocytes take place, thus leading to hair growth [228]. Hair cycle is also linked with melanogenesis as melanin is synthesized only in the anagen phase of hair growth cycle. In the catagen phase, melanogenesis is turned off and remains inactive throughout telogen [229,230].

Clock and *Bmal1* knockout mice showed no dysfunction in hair follicle morphogenesis but both mutant mice displayed a delay in anagen progression, which was more pronounced in *Bmal1* knockout mice [227]. Isolated cultured human hair follicles exhibited a circadian profile in gene and protein of core clock and clock-controlled genes; knockdown of *BMAL1* or *PER1* prolonged the anagen phase [231]. *Bmal1* knockout mice exhibited a reduction on phosphorylated retinoblastoma protein levels (pRB), a cell cycle progression marker, compared to wild type hair follicles, which led to cell cycle arrest [227]. These findings demonstrate the autonomy of hair-follicle clock and suggest an interesting pharmacological target for hair growth [231].

A cyclic and time-dependent growth of hair follicles has been reported *in vivo* as hair growth was faster in the morning compared to evening. When γ-radiation was applied to mice during morning or evening, hair loss was evident in morning-exposed mice since cells were dividing in the morning and therefore were more susceptible to γ-radiation. These time-dependent effects were lost in *Cry1* and *Cry2* double knockout mice [232].

Using hair follicles, an interesting and non-invasive approach to evaluate circadian timing in patients has been reported: By collecting a small number of hair follicles of healthy individuals in controlled environment, circadian clock gene expression was determined [233]. Similar strategy was also used by the same group to evaluate the biological clock of shift workers, which are known to display circadian disruption

[234,235]. In a follow-up study, the *ex vivo* culture of whole hair root through bioluminescence analysis was successfully used to compare circadian timing of healthy patients and patients with severe dementia; no difference on the biological clock parameters was reported [236]. A recent study using beard hair follicle assessed the molecular clock of regular, one-night, and consecutive night shift workers. As expected, shift work affected the clock machinery in comparison to daytime workers; however, substantial variabilities were reported mainly due to a heterogeneous population and lack of standard conditions [237].

Aging

Aging can be defined as the time-dependent decline of biological processes, that affects most living organisms, and can currently be summarized in nine hallmarks: stem cell exhaustion; altered intercellular communication; genomic instability; telomere attrition; epigenetic alterations; loss of proteostasis; deregulated nutrient sensing; mitochondrial dysfunction; and cellular senescence [238]. Regarding the molecular clock of the skin, detectable rhythmic expression was found in two-month old rats, and as rat ages the phase of the clock is more variable when compared to younger mice [81]. Disruption in the molecular clock has been implicated in aging-related alopecia [228] and wound closure [239]. For a deeper view the reader is referred to two reviews which have fully addressed the role of aging in the molecular clock of the skin [70,86].

Other processes

In addition to the above-mentioned processes controlled by the local temporal system in the skin, time-dependent oscillation has also been found in hydration of the *stratum corneum* in humans [83,122]. The molecular mechanism underlying this phenomenon was reported to be dependent on *Clock* since this gene was shown to control the rhythmic expression of aquaporin3 in mouse skin and human keratinocytes [240]. These data demonstrated that skin permeability is higher in the evening and night than in the morning, and thus, it could be relevant for dermatological and cosmetic aspects [83,122]. In addition, wound healing is also regulated by the molecular clock as *Bmal1* knockout mice showed less epithelial coverage and reduced fibroblast proliferation after wound [239]. *Npas2* knockout mice showed faster wound healing *in vivo* compared to control, which was associated with increased proliferation, migration, cell contraction, and collagen synthesis compared to wild type dermal fibroblasts in *in vitro* assays [241].

Other processes of the skin such as blood flow, protection barrier [242-244], surface pH, trans-epidermal water loss [122,123,245], sebum production [123,246,245], skin lipidomic profile [247], and the visibility of wrinkles [248] were shown to be rhythmic along 24 h in skin of healthy individuals (Figure 3).

In addition to circadian rhythmicity, some studies have demonstrated seasonal variations in skin functions. For instance, healthy men and women show seasonal differences in finger temperature and blood flow that were lower in winter compared to summer, despite controlled experimental conditions. Such differences were more pronounced in women than in men as well as in women with primary Raynaud's phenomenon, which is characterized by cold-induced vasospasm of the microvasculature of the fingers [249]. Furthermore, seasonal differences in skin temperature [250], skin hydration, number of corneocytes, TEWL [251,252], skin color and melanin content of pigmented spots [253], sebum production, and skin elasticity [252] have been reported in healthy women. The activity of the antioxidative enzyme, CAT, in the skin, was reported to be reduced and increased respectively in the summer and winter in the same person, while no difference in SOD activity was observed [254]. Additionally, a reduction in *stratum corneum*, lipids, facial cholesterol, ceramides, several fatty acids [255-257], and skin hydration [258] was shown in winter compared to summer [258]. These findings help to explain the increased hand dermatitis [259] and susceptibility to irritant skin agents [260] in winter compared to summer [261,262]. For a deeper view about the role of seasonal rhythms and environmental temperature in skin biology, the reader is referred to a recent review [263]. Despite the well-known reported effects of season rhythms on the skin biology, how the molecular clock of the skin is affected by different seasons, its contribution to skin physiological as well as pathological responses is still completely unknown.

In the current literature, there are some inconsistencies and variation among the studies which are probably due to different experimental designs, number of time points, sampling location sites, and environmental control [86,264]. Despite these limitations, the comprehension of the rhythmic features of the skin [264] could lead to important therapeutic implication in skin-related diseases such as, but not limited to, eczema, psoriasis, acne and wrinkles, and for the application of cosmeceuticals – as addressed by Luber and colleagues [83] – as well as in cancer as detailed in the next pages.

THE MOLECULAR CLOCK IN CANCER

Cancer is a complex disease with hundreds of types and subtypes, that affects most organs and tissues, and is responsible for the death of millions of people worldwide [265]. Understanding how the carcinogenic process takes place is a crucial step to the development of drugs and treatments. Thus far it is accepted that cancer may display up to ten hallmarks, which guarantee its development, proliferation, metastasis, and resistance to treatment [266,267]. The classical hallmarks are: sustained proliferative signaling; evading growth suppressors; activation of invasion and metastasis; enabled replicative immortality; induced angiogenesis, and cell death resistance [267]. In addition, four new hallmarks have been set forth: deregulated cellular energetics; immune system avoidance; tumor-promoting inflammation; and genome instability and mutation [266].

Despite increased evidence that chronodisruption plays a significant role in cancer, chronodisruption has not been appreciated as a possible cancer hallmark; on the other hand, several studies have shown that alteration of the clock gene machinery is indeed associated with the following cancers in mice and/or humans: colorectal [268,269]; liver [270]; oral squamous cell carcinoma [271,272]; breast [66,273-275]; prostate [276,277]; hematologic [278,279]; lung [280]; endometrial [281]; melanoma [93,282-285], and others [65,66]. It is a matter of intense and ongoing research on how the carcinogenic process affects the molecular clock [286]. In fact, it is relevant to mention that the role of biological clock in cancer cannot be generalized as it is dependent on each type of cancer and often varies among its subtypes. More intriguing is the cause-relationship of chronodisruption and the carcinogenic process i.e., whether chronodisruption is the cause or consequence of the carcinogenic process remains unknown.

Despite these open questions, growing evidence regarding the relationship between chronodisruption and cancer development has accumulated and led the International Agency for Research on Cancer (IARC) to classify shiftwork that involves circadian disruption as probably carcinogenic to humans (Group 2A) [287,288]. However – eleven years later – no update on this classification has been made despite the increased knowledge reported in the literature over the last decade.

Cutaneous Melanoma (CM) and the Molecular Clock

Melanoma is the result of uncontrolled proliferation of melanocytes, and it can be subdivided, according to its primary site of location, into cutaneous, mucosal, or uveal. CM – which will be the focus of this review – is one of the most aggressive and treatment-resistant cancers with escalating incidence worldwide. Melanoma represents less than 5% of all cutaneous skin cancer but it accounts for a significant majority of skin cancer related deaths [289-291]. The economic burden caused by melanoma is massive as it comprises $ 3.3 billion (~41%) of a total of $ 8.1 billion – that is the total annual direct cost of all skin related cancers [291,292].

The etiology of CM is multifactorial and includes risk factors such as UV radiation exposure, genetic susceptibility, high nevus density, reduced skin pigmentation, and immunosuppression [293,294]. Caucasian population accounts for the highest incidence while African Americans are the least affected, representing 27.3 and 1.1. per 100 cases, respectively [295]. Men are more susceptible in developing melanoma compared to females [295]. Regarding its primary prevention, it is recommended to avoid indoor tanning and sun exposure during high UV incident hours; in addition, the usage of sunscreen, protective clothes and hat is also strongly encouraged [291,296].

According to high-throughput sequencing, CM is classified into four principal subtypes based on their most prevalent mutations: mutant B-Raf proto-oncogene, serine/threonine kinase (*BRAF*), mutant Kirsten rat sarcoma viral proto-oncogene (*RAS*), mutant neurofibromin 1 (*NF1*), and triple wild type [297]. Subsequent studies using whole-genome sequencing in cutaneous, acral, and mucosal melanoma reported fascinating insights into the molecular biology of these different types of melanoma. Marked differences in the genomic landscape of acral and mucosal melanoma were demonstrated as compared to CM [298], which interestingly displays the highest rate of somatic mutation among all cancers [299], what may represent a therapeutic challenge as well as an opportunity.

It has been suggested an evolutionary progression model of CM, described as follows: mutation in *BRAF* (most common V600E mutation) activates the mitogen-activated protein kinase (MAPK) signaling, leading to limited clonal expansion, and this cascade represents the initiating event of nevus formation. Intermediate stage comprises additional mutations in *NRAS*, other *BRAF* mutations (V600K), and telomerase reverse transcriptase (*TERT*), leading to melanoma *in situ*. As the carcinogenic process advances, subsequent mutations on cyclin dependent kinase inhibitor 2A (*CDKN2A*) leads to G1-

to-S-phase checkpoint disruption, which favors the progression to invasive melanoma. Additional mutations on phosphatase and tensin homolog (*PTEN*) and tumor protein P53 (*TP53*) also contribute to invasive melanoma. Remarkably, point-mutation burden increases from benign, intermediate lesions to invasive melanoma, with a strong correlation of UV-induced mutation signatures; however, genome instability is prevalent in metastatic CM [300]. All the genetic alterations described above result in a complex signaling that overstimulates MAPK, phosphoinositide- 3-kinase (PI3K), protein kinase B (AKT), PTEN, and mammalian-target-of-rapamycin (mTOR) pathways [301].

When primary tumors are detected, the recommended treatment is surgical excision with different safety margins – depending on the Breslow thickness of the tumor [302]; however, tumor recurrence with subsequent metastasis is frequent. Due to the low efficacy of the standard chemotherapeutic regimes used until 2011 (dacarbazine), the overall survival of metastatic melanoma was extremely low (5 months) [303]. However, the development of new therapies that target BRAF, MAPK, and immune checkpoint inhibitor like cytotoxic T-lymphocyte associated protein 4 (CTLA-4), programmed cell death 1 (PD-1) and its ligand PD-L1 has significantly changed the landscape of melanoma treatment. In fact, the combination of these new drugs boosted the overall survival from months into years, thus creating a hallmark in melanoma treatment [301,302,304-307]. Despite the fact that the molecular biology of melanoma is well established [297,298,308], the investigation of clock gene role in CM has been very limited. The first report in 2013 described that clock genes were downregulated in melanoma as compared to normal adjacent tissue; interestingly, the reduced clock gene expression was associated with increased tumor thickness and mitotic level [282]. Following this study, it was demonstrated that RORα and RORγ expression is reduced in melanomas compared to nevi, and as melanoma progresses the expression levels of both proteins decrease. In addition, higher nuclear RORα and RORγ and higher cytoplasmic RORγ levels were correlated with longer overall and disease-free survival; thus, providing an exciting discovery for a putative druggable target in melanoma [309].

In 2016, our group reported that the molecular clock of cultured mouse malignant melanocytes was significantly downregulated compared to normal ones [93], as well as of tumors compared to healthy skin *in vivo* [283], and in human primary and metastatic melanoma compared to human healthy skin [283,284]. Therefore, in CM the molecular clock is significantly impaired, i.e., chronodisrupted, a fact that has been reported in several other cancers [286,310,311].

We have also demonstrated that the molecular clock of malignant melanocytes *in vitro* – although severely less expressed – was more responsive to several stimuli such as visible light [93], UVA radiation [109], temperature [116], and estradiol [110] compared to normal melanocytes. Kiessling and colleagues [285] were able to activate the molecular clock of malignant melanocytes with intratumoral dexamethasone injection, resulting in reduced tumor growth – but not cell death. Taking these findings together an interesting and intriguing speculation arises: Could the activation of the molecular clock by light, radiation, temperature, or hormones lead to cell death or cell cycle arrest? Furthermore, it seems reasonable to speculate that these clock-mediated responses are the result of a residual activity of molecular clock, which could be pharmacologically explored and/or boosted into new drugs and therapeutic options in CM [312]. It should be emphasized that the increased response of the molecular clock was found in *in vitro* studies. Therefore, additional *in vivo* studies aiming to activate the clock with light/radiation and/or temperature are required to consolidate whether the molecular clock might be a new target for CM treatment.

In addition to *in vitro* studies, our *in vivo* data showed an interesting and yet poorly comprehended event: the systemic chronodisruption caused by tumor macro-environment (TMaE) [283]. It is known that tumors are not completely isolated from the organism itself. In fact, tumor-borne molecules like growth factors-, mRNA-, and miRNA-containing micro-vesicles can be found in the bloodstream of cancer patients [313,314]. Classical effects of TMaE are related to cancer-associated cachexia, systemic inflammation, immune system suppression, and altered coagulation [313,314].

Two studies using different experimental models of cancer induction reported interesting results. In the first approach, adenovirus-mediated lung-specific activation and deletion of *Kras* and *Tp53*, respectively, led to a non-metastatic lung adenocarcinoma. In this model, time-dependent hepatic metabolism via a pro-inflammatory pathway was rewired with no change in the clock core machinery [315]. In another model, a non-metastatic breast cancer model, an altered pattern of clock gene expression was detected in the liver of the tumor-bearing mice [316]. These studies, therefore, proved that TMaE can affect the molecular clock of organs distant from the tumor site.

We performed a study of a non-metastatic model of melanoma subcutaneously inoculating B16-F10 cells in C57BL/6J mice. During the two-week long experiment, no metastasis was found, thus making this an excellent scenario to evaluate TMaE effects. We reported that the expression of the molecular clock was significantly reduced in the

melanoma tumor compared to healthy and tumor-adjacent skin [283]. Interestingly, we found that the molecular clock of tumor-adjacent skin displayed an intermediate profile compared to healthy skin from saline-inoculated animals. Thus, these data suggest that tumor-adjacent tissue does not represent a good reference control tissue as it is under the close influence of the tumor, what has been previously discussed [317].

We also reported that tumors show an ultradian profile of melanin synthesis, displaying increased levels of pigment at 9 AM and 9 PM compared to 3 PM and 3 AM in *in vivo* assays. This was– to the best of our knowledge – the first report that tumors display a rhythmic synthesis of melanin [283]. Since increased melanin content is a negative prognostic factor and directly affects the outcome of radiotherapy success [318], we suggest that – if this event is conserved in human CM –chronomodulated radiotherapy could beneficiate patients.

Regarding TMaE, we demonstrated that the presence of a non-metastatic melanoma tumor significantly disrupts the molecular clocks of liver, lungs, and the central clock (SCN). Tissue-specific alterations in the molecular clock were found, but in every analyzed organ, *Bmal1* expression was significantly impaired, which shows that this gene is an interesting cancer "rheostat". In addition, we reported for the first time that TMaE also affects the SCN. Therefore, our data indicate that TMaE, through unknown mechanisms, affects the central and the peripheral clocks. It remains an open question what the consequences of the systemic chronodisruption might be.

Despite the findings described above, an important limitation of the *in vivo* study lies on the fact that B16-F10 inoculation is an artificial xenograft system that does not mimic the carcinogenic process. Thus, further studies using a progression model of cancer development, such as the one described by Masri and colleagues [315], is required to better establish the deleterious influence of a non-metastatic tumor in peripheral and central clocks.

The relationship of the molecular clock with human CM was explored using public data from The Cancer Genome Atlas (TCGA). Since in TCGA database samples were not collected around the clock, analysis of the circadian clock expression is not possible; however, a tool to characterize the status of the molecular clock by using a coexpression matrix with samples collected in a single time point was developed. Using this approach, healthy tissues should display the classic antiphase relationship between *PER* and *BMAL1*. Thus, based on this premise, we found that in metastatic CM the molecular clock displayed reduced amplitudes of antiphase, a fact that was corroborated

with a significant downregulation of clock gene transcripts compared to healthy skin [284]. In addition, we evaluated whether clock genes were a prognostic factor in CM: In fact, high *BMAL1* expression was associated with longer survival. Further analyses demonstrated that high *BMAL1* was also positively associated with stronger immune system activation.

Increased immune system activation was correlated with a deficiency in base-excision repair (BER) pathway in high *BMAL1* expressing tumors with consequent increase of total mutational burden and neoantigen presentation. All these events contributed to the increased immune system activation in tumors expressing high *BMAL1* transcripts [284]. An interesting result from this study was a positive correlation of high *BMAL1* expression with T-cell inhibitors and exhaustion markers. Remarkably, there is evidence that patients with high *BMAL1* expression in pretreatment biopsies demonstrated improved response to anti-PD1 immunotherapy in comparison to patients expressing low *BMAL1* levels [284]. Therefore, *BMAL1* could be used, along with other biomarkers, to discriminate patients that will benefit from immunotherapy related therapeutic regimes. In fact, the search for biomarkers that will help decide whether immunotherapy should or should not be implemented is urgently needed since only a subset of patients benefit from immunotherapy [319,320].

Furthermore, a recent study has provided an advance in cancer treatment through molecular clock interaction. REV-ERBs agonists were reported to be effective in a wide variety of tumors – including melanoma – demonstrating a low toxicity and higher selectivity for malignant cells. Such antitumor activity was reported to be dependent on the inhibition of *de novo* lipogenesis and autophagy [321].

Having in mind that melatonin is a major output signal of the mammalian central clock, one may suggest that melatonin might be an important player in the carcinogenic process of CM. This argument resides on the fact that melatonin and its metabolites protect against the deleterious effects of UV radiation [175,178], as well as display direct antiproliferative effects on melanoma growth [169,322-325]. In high-grade glioma patients, an interesting approach evaluated the expression ratio between *ASMT* and *CYP1B1*, genes that encode enzymes responsible for melatonin synthesis and degradation, respectively. Low *ASMT*:*CYP1B1* ratio value, which suggested decreased melatonin levels, was associated with poor survival [326]. Although this index has not been evaluated in cutaneous melanoma, it is an interesting strategy to be investigated for prognosis and therapeutical marker of this skin cancer. Despite the well-known effects of

melatonin in cutaneous melanoma, how this hormone affects the molecular clock of melanoma cells is still a matter of investigation.

Taken altogether the *in vitro*, *in vivo*, mouse and human studies reported in the literature regarding CM clearly demonstrate that the molecular clock is an interesting and exciting field of study, but still poorly comprehended and, therefore, its putative clinical and therapeutic implications remain largely unexplored. Thus, further studies are necessary to better understand the role of the molecular clock in tumor development, progression, and metastasis.

CONCLUSIONS, OPEN QUESTIONS, AND FUTURE DIRECTIONS

Our knowledge regarding the role of the molecular clock in the skin has significantly increased over the past decade. Currently, we know that several functions of the skin are rhythmic, which could be pharmacologically explored; however, the specific part played by the molecular clock in each skin cell type is still largely unknown as most of the studies have used global knockout animals. To provide important advancements in this field, the deletion of core clock genes in a specific skin cell type is required, which could be achieved through the usage of Cre-Lox systems. Another important limitation that reduces the scientific progress is the lack of standard experimental conditions among different laboratories, such as: light exposure (light source, wavelength, and irradiance); period of the day in which animals are exposed to light; maintenance in neutral temperature; and the time or period that experiments were carried out.

In addition to the physiological approach, it is also necessary to understand the participation of the timing system in diseases that affect the skin, what could lead to therapy improvement. Within this line, the participation of the molecular clock in cancer is even less investigated. Since the molecular clock is important to regulate in a time-dependent fashion the physiology of the whole body, in addition to the fact that this system is disrupted in cancer, we speculate that clock machinery disruption could represent a new cancer hallmark. An important question that has not been answered yet, but when addressed, will bring significant advance, is: does chronodisruption take place in early, intermediate, or advanced stages of melanoma? Furthermore, how the loss of proper temporal control of the physiological processes affects the development of the hallmarks of cancer. In this approach, understanding the function of the molecular clock in cancer could lead to important breakthroughs, which could be translated into clinical gain to CM patients.

Another hypothesis raised by our group is based on the fact that the skin possesses complex – and yet not fully known – photosensitive [93,94,109] and thermosensitive systems [116] based on opsins [151] whose function may be altered by the carcinogenic process. Ultimately, our hypothesis suggests that melanomas are more sensitive to external and internal cues, i.e., light, temperature and hormones than normal melanocytes; this altered sensitive system then would feed the disrupted clock with external timing, in a SCN-independent fashion. In fact, Masri and colleagues [315] have clearly demonstrated the potential consequences of a tumor to the biological clock of the liver, acting as an endogenous circadian organizer. Therefore, all these tumor-induced alterations could favor cancer development, progression, and then, they could be pharmacologically targeted.

Taken altogether, understanding the participation of the molecular clock and the processes and mechanisms it regulates is an active, ongoing, complex, and fascinating field that can provide important knowledge regarding skin biology at both the physiological and pathological levels. Therefore, comprehending how time affects our skin will expand our knowledge into clinical application and will result in increased life expectancy and/or quality.

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CONFLICT OF INTEREST

All authors state no conflict of interest that could have impacted the development of this study.

AUTHOR CONTRIBUTIONS

de Assis, L.V.M. designed the manuscript's outline and content, reviewed the current literature, and drafted the first version of the manuscript. Moraes, M.N. and Castrucci, A.M.L. critically revised the manuscript and provided substantial contribution and improvements to the manuscript. All authors have approved the definitive version of the manuscript and agreed to be accountable for all aspects of the study in ensuring that questions related to the accuracy or integrity of any part of the study are appropriately investigated and resolved. Each person designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Figure

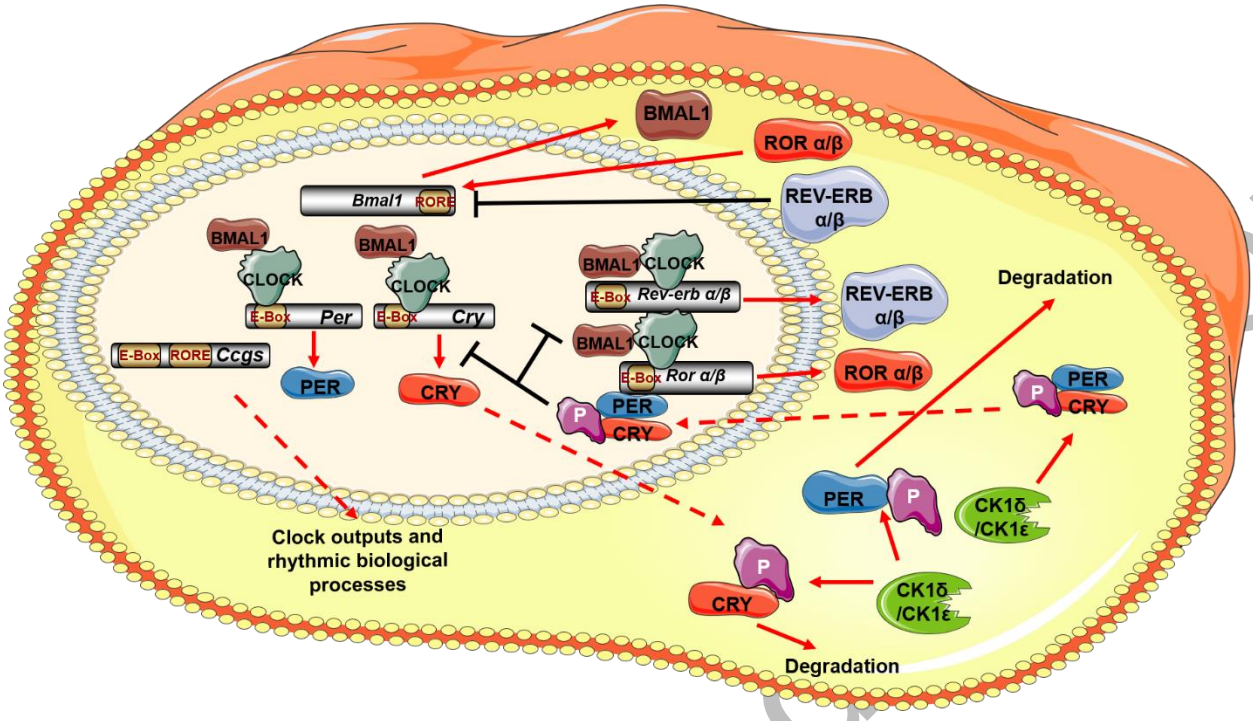

Figure 1. The molecular mechanism of the clock genes in mammals. Genes and proteins that comprise the molecular clock are expressed in almost every mammalian cell. The full cycle takes 24 h to be completed and it is the molecular basis of how the organism keeps track of the time. Please refer to the text for a detailed description.

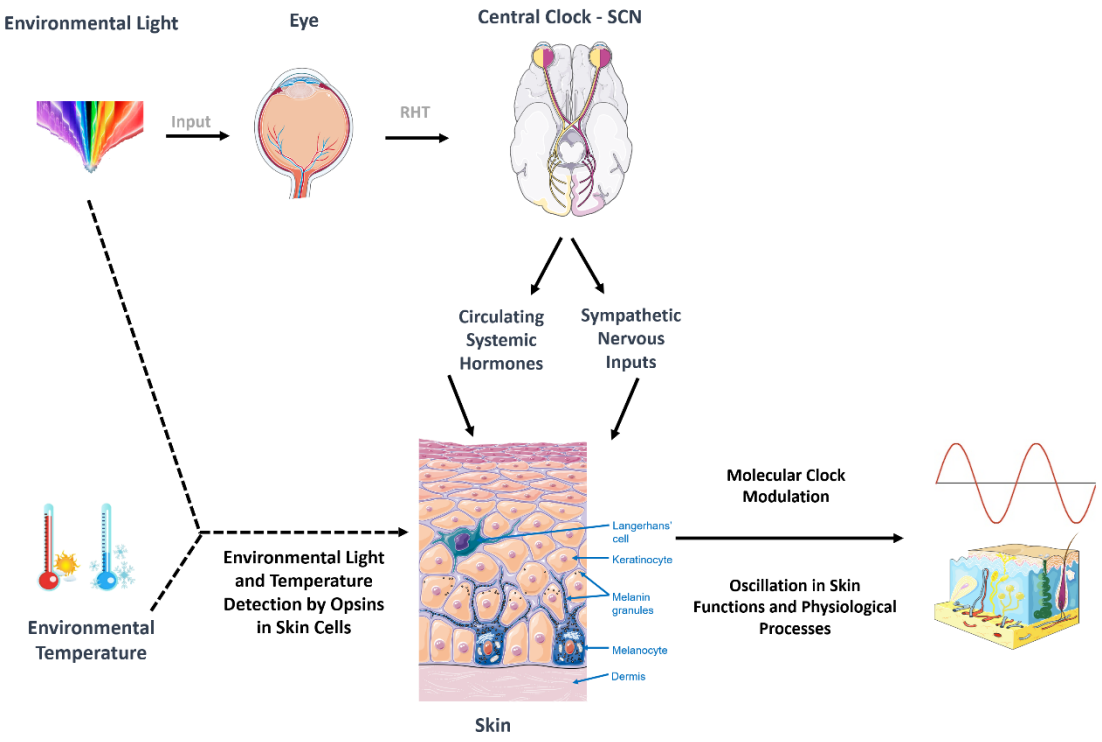

Figure 2. Circadian organization of the skin. Environmental light information is detected by melanopsin-expressing retinal ganglion cells which translate it into electrical stimuli sent to the central clock (SCN) via the retinohypothalamic tract. Through a glutamate-dependent pathway, the molecular clock of the SCN is reset and a new cycle of gene transcription and translation, as described in Figure 1, takes place. Upon synchronization of the SCN, this temporal information is shared with other brain regions that control several important functions in the organism. Regarding the skin, both sympathetic inputs and systemic hormones modulate its molecular clock, thus aligning skin timing with other organs and systems. The molecular clock of the skin regulates biological processes in this organ in a time-dependent manner. The dashed lines represent a phenomenon that requires *in vivo* validation. However, i*n vitro* data clearly show that skin cells, i.e., keratinocytes, dermal fibroblasts, and melanocytes, express light– as well as – temperature-sensitive sensors, namely opsins, leading to modulation of the molecular clock.

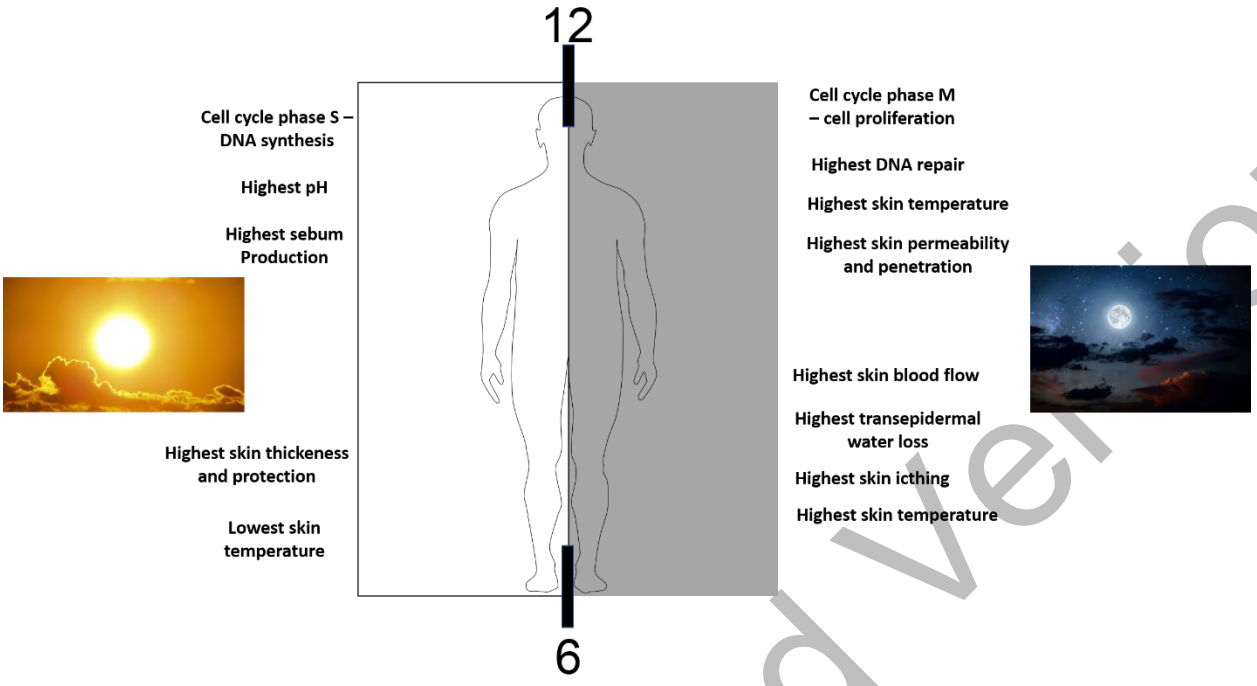

Figure 3. Circadian functions in human skin. Several skin functions display maximal and minimal values along 24 h, controlled by the molecular clock of the skin. Please, refer to the text for detailed information.

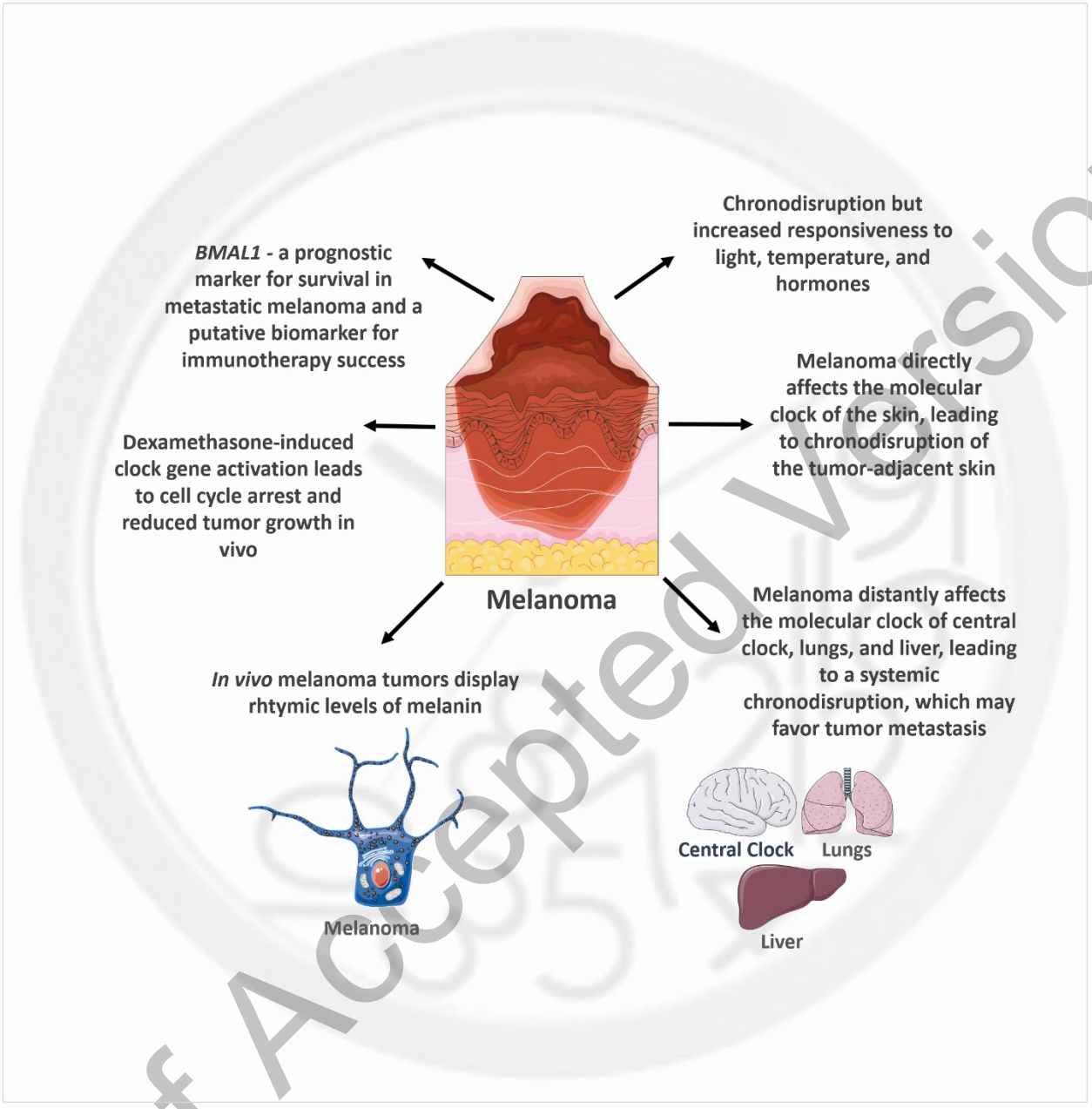

Figure 4. The role of the molecular clock in cutaneous melanoma. The above figure should be read clockwise. *In vitro, in vivo*, and human data banks clearly demonstrate that in primary and metastatic cutaneous melanoma (CM) a chronodisruption scenario is installed. It is a matter of investigation the cause-relationship, i.e., whether chronodisruption is an early, intermediate, or late process in CM development and progression. In the skin, melanoma tumor leads to a microenviroment chronodisruption in tumor-adjacent skin that represents an intermediate stage between malignant and healthy tissue. Through its tumor macroenvironment effects (TMaE), still unknown melanoma-borne molecules leak from the encapsulated tumor, and reach distant molecular clocks in the SCN, lungs, and liver. We suggest that these alterations may favor the establishment of tumor metastasis, which still require experimental validation. Melanoma tumors, although displaying a chronodisrupted clock machinery, still exhibit an oscillatory profile in

melanin content, which may favor tumor resistance to radio- and chemotherapy regimens. *In vivo* data demonstrate activation of the molecular clock of melanoma cells upon synchronization by dexamethasone, which leads to cell cycle arrest and consequent reduced tumor growth. Lastly, *BMAL1*, a clock gene, is a prognostic marker for survival in human metastatic melanoma, and it may be used as a biomarker for immunotherapy success.