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The effect of white light on normal and malignant murine melanocytes: A link between opsins, clock genes, and melanogenesis

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ABSTRACT

The skin possesses a photosensitive system comprised of opsins whose function is not fully understood, and clock genes which exert an important regulatory role in skin biology. Here, we evaluated the presence of opsins in normal (Melan-a cells) and malignant (B16–F10 cells) murine melanocytes. Both cell lines express *Opn2*, *Opn4* – for the first time reported in these cell types – as well as *S-opsin*. OPN4 protein was found in a small area capping the cell nuclei of B16–F10 cells kept in constant dark (DD); twenty-four hours after the white light pulse (WLP), OPN4 was found in the cell membrane. Despite the fact that B16–F10 cells expressed less *Opn2* and *Opn4* than Melan-a cells, our data indicate that the malignant melanocytes exhibited increased photoresponsiveness. The clock gene machinery is also severely downregulated in B16–F10 cells as compared to Melan-a cells. *Per1*, *Per2*, and *Bmal1* expression increased in B16–F10 cells in response to WLP. Although no response in clock gene expression to WLP was observed in Melan-a cells, gene correlational data suggest a minor effect of WLP. In contrast to opsins and clock genes, melanogenesis is significantly upregulated in malignant melanocytes in comparison to Melan-a cells. *Tyrosinase* expression increased after WLP only in B16–F10 cells; however no increase in melanin content after WLP was seen in either cell line. Our findings may prove useful in the treatment and the development of new pharmacological approaches of depigmentation diseases and skin cancer.

1. Introduction

The skin is the first barrier between the organism and the external environment and it is subject to frequent daily oscillation of environmental temperature and light. These two factors represent an important source of possible damage to skin cells [13,15,17,19,70]. Since oscillation in the amplitude of these factors can be predicted, a system able to anticipate these cyclic oscillations is crucial for the organism homeostasis.

At the core of this temporal system lies the molecular clock gene machinery [11,12,18,59,65]. *Clock* (*Circadian Locomotor Output Cycles Kaput*) and *Bmal1* (*Aryl hydrocarbon receptor nuclear translocator-like protein 1*) represent a positive component while *Per* (*Period*) and *Cry* (*Cryptochrome*) compose the negative limb of a system of interlaced feedback loops. The oscillation in clock gene expression and subsequently the protein levels are crucial to keep track of time through a well-described auto-regulatory feedback loop of transcription factors. BMAL1 forms a heterodimer with CLOCK or NPAS2 (in the suprachiasmatic nucleus, SCN), which activates the transcription of *Per1*, *Per2*, and *Per3*, and *Cry1* and *Cry2* by binding to a specific DNA element named E-box in the promoter of the target genes. Once PERs are in the cytoplasm they dimerize with CRY1 and CRY2 which, after being phosphorylated by casein kinase 1δ (CK1δ) or CK1ε, are targeted toward the nucleus to inhibit the transcriptional activity of BMAL1/CLOCK heterodimer. Another loop of regulation is also exerted through E-box activation: CLOCK/BMAL1 activates *nuclear receptor subfamily 1, group D, member 1/2* (*Rev-Erbα/β*) and *RAR-related orphan receptor alpha/beta* (*RORα/β*) expression. After being translated, REV-ERBα/β competes with RORα/β for the orphan receptor response element (RORE) sequence present in *Bmal1* promoter: REV-ERBα/β inhibits while RORα/β stimulates *Bmal1* expression. To terminate the repression phase and to restart a new cycle of transcription, the degradation of PER and CRY is required. Such event is carried out by CK1δ or CK1ε that target PER for ubiquination whereas CRY is phosphorylated by 5′-AMP-activated protein kinase catalytic subunit alpha-1 (AMPK1) and dual specificity tyrosine-phosphorylation-regulated kinase 1 A (DYRK1A) (for review see [11,12,18,59,65]).

In mammals, the SCN is the central oscillator [71] responsible for controlling the temporal expression of clock genes and clock-controlled genes (CCG) in peripheral tissues, and ultimately align the

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entire organism under a single timing [11,59]. The light and dark cycle (LD) is the most important environmental timing clue, *Zeitgeber*, in mammals; this information is interpreted by a subset of retinal ganglion cells – that express melanopsin, OPN4 – into electrical stimuli that reach the SCN through the retinohypothalamic tract, which then entrain the SCN [63,64].

The presence of clock genes in peripheral tissues was first reported in 1998 in isolated mammalian fibroblasts [1], and two years later in the skin [95]. In 2001, the expression of clock genes in human oral mucosa and skin was reported to be cyclic, and an association between *Per1* and cell cycle was reported [9]. Since then few studies have investigated the effect of several stimuli [75,83], among them the white light [84], on the regulation of clock gene expression in murine and human skin cells. Recently, the important role of clock genes in the regulation of skin pigmentation response was reported [28], and currently it has been proposed that the skin cells – keratinocytes, melanocytes, and fibroblasts – display distinct clock gene machineries that comprise a local multioscillatory circadian system [75].

Several features have been reported to oscillate in a circadian manner in the skin such as: blood flow, protection barrier [92], transepidermal water loss, surface pH, and temperature [93]. For instance, DNA repair has been shown to oscillate in murine skin with a maximal activity in afternoon/evening, which coincides with the onset of mouse locomotor activity [22]. Recently, mice harboring mutation on *Clock* showed decreased hydration of the stratum corneum, what was linked to reduced aquaporin 3 expression (*Ap3*) in the epidermis [55].

In addition to clock gene machinery, murine and human skin [43,85], as well as cultured melanocytes [24,37,51,58,85], display an interesting photosensitive system comprised of opsins; however, the role and functionality of this photosensitive system in regulating local physiological processes are still poorly understood but it began to be recently addressed [3,4,87].

Melanocytes are specialized cutaneous cells which produce and store melanin, a pigment produced in lysosome-like structures named melanosomes. Shortly, tyrosine is oxidized by an enzymatic complex formed by tyrosinase and dopa oxidase, resulting in dopaquinone. In the presence of high concentration of thiol groups pheomelanin formation is favored; on the contrary, dopaquinone is transformed in leucodopachrome, which then generates a mixture of 5,6-dihidroxyindol (DHI) e 5,6-dihidroxyindol-2-carboxylic acid (DHICA). The last reactions catalyzed again by tyrosinase produce eumelanin [82], which may be transferred to adjacent keratinocytes where it forms a perinuclear shield protecting the cells against the deleterious effect of ultraviolet (UV) radiation and white light. Even though the classical and well-known protective role of melanin is well established [81], emerging evidence is showing that melanin may participate in DNA damage through reactive oxygen (ROS) and nitrogen species (RNS) in response to UVA radiation and white light [14,67]. Whether melanin has as protective and/or a carcinogenic role in skin cancer has yet to be established. Despite this open question, it has been demonstrated that melanocytes respond to white light pulse (WLP) with increased melanin content through immediate pigment darkening (IPD), persistent pigment darkening (PPD), and delayed tanning (DT) [54,73,80].

Based on the above-mentioned literature, we hypothesized that the malignant transformation alters the melanocyte photosensitive system. To test this hypothesis we investigated the response of opsins, clock genes, and melanogenic machinery evoked by WLP in normal, Melan-a, and malignant, B16–F10, murine melanocytes.

2. Material & methods

2.1. Cell culture

Melan-a or B16–F10 cells were cultured in 75 cm^2 flasks with RPMI 1640 medium containing phenol red (Atená, Campinas, SP, Brazil), supplemented with 14.3 mM $NaHCO_3$, 15 mM HEPES, 10% fetal bovine serum (FBS) (Atená, Campinas, SP, Brazil), and 1% antibiotic/antimycotic solution (10,000 U/mL penicillin, 10,000 μg/mL streptomycin, and 25 μg/mL amphotericin B, Life Technologies, Carlsbad, CA, USA). The pH was adjusted to 7.2, and the cells were kept at 37 °C with 5% CO_2. The maintenance of the cell cultures and the experiment setup were carried under ambient lighting. The cells were kept during six days in complete darkness in order to free run.

During the experiments the cells were maintained in RPMI 1640 without phenol red (Atená, Campinas, SP, Brazil or Life Technologies, Carlsbad, CA, USA) in 25 cm^2 flasks at 37 °C with 5% CO_2. FBS was reduced to 2%, and the medium was supplemented with 10^{-7} M retinal (*all-trans* Sigma-Aldrich, St. Louis, MO, USA). Phorbol 12-myristate 13-acetate (TPA, Sigma-Aldrich, St. Louis, MO, USA) at 200 nM was always present in Melan-a cell media, as it is required for proper cell growth [5]. Cell manipulation during the experiments was carried out under red dim light (7 W Konex bulb and Safe-Light filter GBX-2, Kodak, Rochester, NY, USA). During the experiments no cell handling or feeding was carried out.

2.2. White light stimulation

Melan-a or B16–F10 cells were seeded at the initial density of 10^6 and 10^5 cells, respectively. The cells were divided into 2 groups: (1) control group, kept in DD throughout experiment and (2) experimental group kept in DD for six days, exposed to a 15 min WLP at the beginning of the 7th day (420 to 750 nm, 95.18 μW/cm^2, 650 lx), and returned to DD until the end of the experiment. No UVA or UVB radiation was detected in the white light spectrum (UV Detector, Vilver Lourmat, Marne-la-Vallée, France). Total RNA was extracted 24 h after the WLP, and then every 6 h for 18 h. For melanin quantification assay, cells were kept as above, but the extraction took place 24 (ZT 24) and 36 (ZT 36) hours after the WLP.

2.3. Total RNA extraction and reverse transcriptase PCR (RT-PCR)

The medium was removed and Tri-Reagent (Ambion, Grand Island, NY, USA) was added directly to the cells. The lysate was subject to the manufacturer's suggested procedure. In order to remove genomic DNA contamination, the samples were treated with DNAse I (turbo-DNase, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration and quality (OD_{260}/OD_{280}) were determined in a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE, USA). One to four μg of total RNA was subject to reverse transcriptase reaction using random primers and Superscript III, in addition to the reagents recommended by the enzyme manufacturer (Life Technologies, Carlsbad, CA, USA).

2.4. Quantitative PCR (qPCR)

Quantitative PCR was performed with the products of reverse transcription using oligopeptides spanning introns, designed and synthesized by IDT (Coralville, IA, USA) or Life Technologies (Carlsbad, CA, USA), based on sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank). All primers exhibited efficiency between 86% and 120%. The access number of each gene, the respective primer sequences and the concentrations are shown in Table 1.

The qPCR reactions were performed through two different protocols: multiplex for simultaneous analysis (*TaqMan*®) and *SYBR® GreenER™*. The *TaqMan*® solutions contained *Per1* and *Clock*, or *Opn2* and *Bmal1*, primers and fluorescent probes (Table 1), Supermix 2× (Bio-Rad Laboratories, Hercules, CA, USA, or Life Technologies, Carlsbad, CA, USA), supplemented to final concentrations of 400 μM dNTPs, 6 mM $MgCl_2$, and 0.1 U/μL Platinum Taq DNA polymerase (Life Technologies, USA). Each experimental cDNA was run in triplicates in 96 well plates. The assays were performed using i5