

Clock gene expression studies in human skin biopsies

Ph.D. thesis

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Pécs
2013.

1. INTRODUCTION

1.1. Circadian rhythm and clock genes

A biological rhythm is one or more biological events or functions that reoccur in time in a repeated order and with a repeated interval between occurrences. Circadian biological rhythms show a temporal pattern which tracks light/dark cycles resulting from Earth's rotation around its own axis (circa- approximately, diem- a 24 h day). These daily rhythms have been widely observed in prokaryotes, eukaryotes, plants, fungi, animals and humans. The mammalian circadian timing system generates daily rhythms that are crucial for normal behavior and various physiological processes, such as sleep-wake cycle, hormone secretion, core body temperature, metabolism and cell cycle control. Within one period, the actual stage (phase) of the rhythm can be easily shifted with a proper environmental stimulus (Zeitgeber: german „time giver”). The most important Zeitgebers are light, temperature, food, physical activity, sleep-awake- and social stimuli. Besides melatonin, insulin, glucocorticoids, and catecholamins act as endocrin-metabolic Zeitgebers. In mammals the circadian system consists of a central and numerous peripheral clocks, which are regulated by positive-, and negative feedback loops. In mammals the key regulator is located within the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. The centrally generated systemic rhythms coordinate molecular clocks in peripheral organs. In the last 20 years, several clock genes and proteins have been identified and implicated in the molecular regulation of circadian rhythms both in the SCN and in peripheral tissues. The mammalian molecular clock is regulated by transcriptional and post-translational feedback loops. The positive regulators of the circadian clock are the „*circadian locomotor output cycles kaput*” (CLOCK) and the „*brain and muscle ARNT-like*” (BMAL1) proteins that heterodimerize, enter the nucleus and induce expression of *per* and *cry* genes by binding to their promoters at E-boxes. The cryptochrome (CRY1 and CRY2) and period homolog (PER1, PER2 and PER3) clock proteins act as negative regulators by forming a complex to inhibit CLOCK/BMAL1 mediated transcription in the nucleus, thus also inhibiting their own transcription (**Fig. 1.**). It has also been shown that the CLOCK/BMAL1 complex directly regulates cell cycle genes such as *wee1* (G2/M transition), *c-myc* (G0/G1 transition) and *cyclin D1* (G1/S transition). The diversifying connection between cell cycle and the circadian clock have been verified by numerous studies.

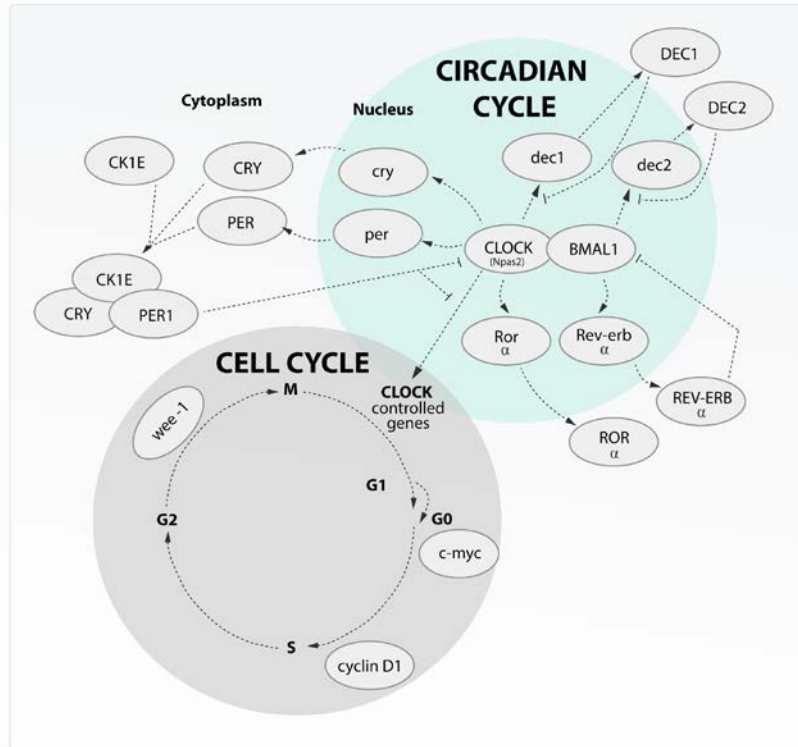


Figure 1.

Schematic representation of the mammalian circadian clock mechanism

Circadian rhythmic regulations are involved also in the repair of DNA damage and in apoptosis control. Recent study indicated that among the different DNA repair mechanisms, nucleotide excision repair is tightly controlled by the circadian clock. Studies have shown that both intrinsic and extrinsic pathways of apoptosis seem to interfere with the circadian system.

1.2. Clock genes and carcinogenesis

Looking at the diversity of mechanisms influenced by the circadian system, it is not surprising that the impairment in the clock function has been implicated in cancer formation in numerous studies. Epidemiological data suggested that the disruption of circadian rhythms in behaviour is associated with a higher risk for cancer formation. Studies of human breast and colon cancer have revealed that disruption of circadian rhythms primarily in shift workers increases risk of disease development. Based on numerous similar experimental evidences, shift work is considered by the WHO (International Agency for Research on Cancer, IARC) as “probably carcinogenic to humans”. From the molecular aspect, several studies suggested tumor suppressor functions for circadian rhythms. Some components of the molecular clock might be directly involved in tumor suppression (*per1, per2*), while the roles of other clock genes in this process are less clear. Experiments on human tumorous biopsies consistently showed down-regulated clock gene expression in various cancer types in comparison to

adjacent non cancerous biopsy, *e.g.* in breast-, endometrial-, prostate-, colon-, liver, head and neck squamous cell, lung cancer. It is important to emphasize that descriptive data on clock gene expression gained from human tumor biopsies do not answer the question if the finding is a cause or consequence of malignant transformation. Epigenetic down-regulation could also be a result of altered neuro-humoral or metabolic environment caused by antitumor reactions (local or systemic) of the body.

1.3. Circadian rhythms in the skin

Skin is a barrier organ continuously exposed to diurnal changes in environmental conditions. Rhythmic pattern in some biophysical and physiological parameters of human skin are well known (*e.g.* sebum production, barrier function). **Table 1** depicts the publications on the relationship of the skin and the circadian system. In conclusion, the skin contains functional peripheral clocks, which make it an ideal model to study the relationship of circadian timing and tumor development.

1.4. Melanoma and the circadian system

The effects of circadian disruption on the frequency of SCC or melanoma formation have not been documented in animal experiments. The epidemiological studies available about the relationship between skin tumors and circadian disruption are ambiguous. A higher risk of melanoma was reported in airline personnel related to their job, but the role of the lifestyle in the increased risk currently remains unclear. Other studies on shift workers and on flight attendants question the correlation between circadian disruption and skin tumor incidence. More recently, 44% reduction in melanoma risk was reported among night shift workers. The lack of increase in the risk for melanoma incidence under circadian disruption together with a greater aggressivity of melanoma growth under such conditions is rather interesting. Growth and proliferation seem to be promoted only in already transformed skin cells with impaired apoptotic response to the metabolic and neurohumoral dysregulation of circadian disruption. Since no particular hormone has been proven until now to play an exclusive or indispensable role in melanoma development, uncovering details on the circadian regulation of cellular metabolism in the skin may be more promising than the search for new humoral factors.

Table 1. Expression of circadian clock genes in the skin

Publication date	Sample	Results
2000.07.	Keratinocyte- (HaCaT), melanocyte-, fibroblast-, melanoma A375 cell lines	Evidence for expression of <i>clock</i> and <i>per1</i> mRNAs and proteins in various skin cell types.
2001.02.	Human skin biopsies	Daily rhythm in the expression of <i>clock</i> , <i>tim</i> , <i>cry1</i> , <i>bmal1</i> , <i>per1</i> <i>in vivo</i> .
2002.08.	Human keratinocyte cell lines	Low-dose UVB irradiation induces altered expression of <i>clock</i> , <i>per1</i> , <i>bmal1</i> .
2008.11.	C57BL6 wild type and <i>Cry1^{-/-}Cry2^{-/-}</i> mice	Oscillation of skin clocks abolished after SCN ablation.
2009.07.	C57BL6 wild type, <i>Clock^{-/-}</i> , and <i>Bmal1^{-/-}</i> mice	CLOCK-regulated genes are modulated in phase with the hair growth cycle. Delay in anagen phase is observed in case of mutant mice. Upregulation of p21 in <i>Bmal1^{-/-}</i> mice.
2010.09.	HaCaT keratinocytes	<i>In vitro</i> rhythmic expressions of circadian clock genes and clock controlled genes, involved in epidermal physiology (<i>Insig2a</i> , <i>c-myc</i> , <i>Ldl receptor</i> , <i>Hmgcr</i>).
2011.11.	Male outbred SKH-1 mice	The xeroderma pigmentosum group A (XPA) protein expression and the excision repair rate show daily rhythmicity in the skin. Exposure to UV radiation at 4:00 AM caused cca. five fold higher multiplicity of skin cancer (invasive squamous cell carcinoma) than mice exposed at 4:00 PM.
2011.11.	<i>Bmal1^{-/-}/K5-SOS</i> mice	Fewer neoplastic lesions on the skin of <i>Bmal1^{-/-}/K5-SOS</i> mice in all stages of cancer development if compared to K5-SOS.
2012.05.	Human melanocyte, keratinocyte and fibroblast cultures	<i>In vitro</i> rhythmic expression of the clock genes with different amplitudes of oscillation in each cell types.
2012.05.	Human epidermal biopsies from healthy volunteers	Hundreds of genes show daily fluctuations in their mRNA levels in the human epidermis <i>in vivo</i> (microarray results).

2. AIMS

Several *in vitro* studies have proved the role of circadian system in cell cycle-, DNA repair mechanisms and apoptosis. The impaired function of any of these may lead to cancer formation. Clinical studies were carried out in which biopsies were obtained from the tumor and the adjacent non-tumorous tissues and the measured clock gene mRNA expression data was compared. The results of the experiments consistently showed down-regulated clock gene expression in various cancer types in comparison with adjacent non cancerous biopsy. Many

studies have assumed that decreased expression of *per* gene may lead to tumorformation as its tumorsuppression function is proven at molecular base. However analyzing these results we have to consider that only limited data has been documented on cellular composition of these clinical samples and the reduction in mRNA content differences was modest. Consequently, the measured decreased mRNA contents at the tissue level could be simply caused by altered cellular composition of the tumorous biopsy if compared to the paired adjacent non-tumorous biopsy. To show a clearer picture, transcriptional alterations not only in cancer cells but also in adjacent non-tumorous cellular compartments within the tumorous tissue (e.g. lymphomonocytotic infiltration) need to be examined simultaneously. Based on its heterogenous composition and ease of access, skin seems to serve as a good model for such histomorphological studies.

- (1) At the beginning of our research there was only one human study, which proved the presence of clock genes and their rhythmicity in human skin samples. No data was available on clock genes expression in case of skin diseases (skin tumor in our case). We planned to take biopsies from melanoma malignum and the adjacent non-tumorous skin to determine clock gene expression both at mRNA and protein levels in the samples.
- (2) Several studies have been conducted previously in various cancer types (except melanoma), where clock genes expression of the tumor biopsy was compared to the adjacent non cancerous biopsy. Most human studies on tumorous biopsies with heterogenous cellular compositions provide cca. 0.5 fold difference in the mRNA amounts of clock genes between paired tumorous vs. non-tumorous tissue samples. Similar comparison was planned at mRNA and protein levels.
- (3) To determine overall survival in cancer patients prognostic factors are evaluated. In our experiment we planned to seek for correlations between the clock genes expression and clinico-pathological features in the melanoma patients.
- (4) To clarify that the difference observed in clock gene expression between tumorous and non-tumorous samples is may be due to altered cellular composition we planned to gain samples from benign melanocyter lesion (naevus) and it's adjacent healthy skin. The presence of clock genes expression was examined first and afterwards the available data on melanocytic lesions and adjacent skin were planned to be compared.

3. PATIENTS AND METHODS

Tissue samples from 32 patients (mean age 63, minimum 24, maximum 88) who underwent surgery due to suspected melanoma were collected at PTE ÁOK Dermatology and Oncodermatology Department. There were no signs of distant or sentinel lymphnode metastasis in any of the patients. 3 mm dermatological punch biopsies were obtained from the primary melanoma which was removed surgically from each patient between 9:00-12:00 a.m. Non-cancerous samples were collected either from adjacent skin in average of 2 cm from the melanoma. In addition, non-cancerous samples were collected also from naevi and adjacent skin 1 cm from the naevus in 10 additional patients without malignant disease. After removing the subcutis from all the punch biopsies with a sterile blade, the samples were immediately homogenized in TRI Reagent (SigmaT9424) and stored at -70°C until processing for RT-PCR analysis. Tumor diagnosis and classification was confirmed by histopathological analysis of the whole excised skin samples. Cell composition of all specimens was determined according to routine pathohistodiagnostic protocol of the University of Pécs (haematoxylin-eosin staining, S-100, HMB45A, Melan-A immunostaining, tyrosinase histochemistry). Furthermore, CLOCK and PER1 immunopositivity was tested in both malignant and non-malignant sample blocks. According to the routine histopathological examination, 26 out of 32 patients were diagnosed with melanoma malignum. Four of the samples could not be evaluated with RT-PCR because of high melanin contents which interfered with the fluorescent measurements. According to the measured tumor thickness and the American Joint Committee on Cancer Staging we have found that out of the 22 melanoma samples 3 samples were *in situ* melanoma ($T_{in situ}$), 7 were <1 mm (T_1), 7 were 1-2 mm (T_2), 4 were 2-4 mm (T_3), and one was thicker than 4 mm (T_4). Most of the processed melanoma samples were superficial spreading melanomas (n=18), two were lentigo maligna melanomas, one was acrolentiginosus melanoma malignum, and one was nodular melanoma malignum. Out of the ten naevi samples 7 were histologically proven as being compound-, and 3 as junctional naevi. To determine the mRNA expression of the clock genes real-time RT-PCR was used. Total RNA was extracted according to the specifications of the TRI-reagent's manufacturer (SigmaT9424). Absorbance was measured at 260 nm with Ultrospec 2100 instrument. Real-time PCR was run using 3 μ L cDNA solution per 15 μ L reaction volume with Applied Biosystems' TaqMan gene expression assays for *per1* (Hs00242988_m1), *per2* (Hs00256143_m1), *clock* (Hs01546767_m1), *cry1* (Hs01597805_g1); and also for internal reference genes *β -actin* (Hs99999903_m1) and *hprt1* (Hs99999909_m1) which show little or no variation between healthy and tumor skin samples. Furthermore immunohistochemistry

was performed with clockproteins (CLOCK and PER1), which selection was based on our RT-qPCR results. To quantify differences in clock gene mRNA contents of the obtained biopsies, the $\Delta\Delta C_T$ method was used after control measurements for reaction efficiency normalizations. To quantify differences in clock protein contents of the obtained biopsies, the number of cells with strong nuclear immunopositivity was counted and averaged in three different sections of the biopsy. In each section, three different fields were photographed. The cell counting was performed on non-edited digital color images, without knowing the histopathological result. Strong nuclear immunopositivity was considered in cells with brown colored nuclei masking the blue counterstaining (haematoxylin) completely (**Fig. 2**). Differences in clock gene expression between biopsy types (melanoma, adjacent skin, naevus, healthy skin) or between clinicopathological categories of melanoma were evaluated with *Student's* two sample test (two-tailed, with unequal variances). All statistical analysis were performed using SigmaStat for Windows Statistical Software, version 3.5 (Systat Software Inc., San Jose, California), where $p < 0.05$ was considered to be statistically significant.

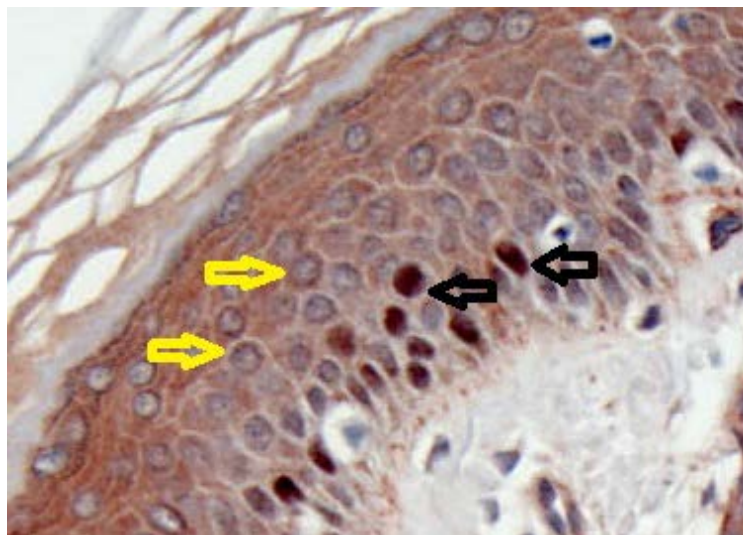


Figure 2.

Immunopositive (black arrow)-, and immunonegative (yellow arrow) cells

4. RESULTS

Expression of *Per1*, *Per2*, *Clock* and *Cry1* in Skin Biopsies of Melanoma Patients

Levels of clock gene mRNAs showed a decrease in melanoma tumors when compared to adjacent non-tumorous skin tissues (**Fig. 3A**). On average, *per1*, *per2*, and *clock* showed significantly decreased levels of expression (p values were 0.020, 0.034, 0.020, respectively), while decrease in *cry1* expression proved not to be significant ($p=0.081$). The number of PER1 or CLOCK nuclear immunopositive cells in our skin samples showed a clear decrease in melanoma tumors compared to adjacent skin ($p<0.001$, **Fig. 3C**). The majority of PER1 or

CLOCK nuclear immunopositive cells were mostly keratinocytes of the epidermis both in the tumor and the adjacent skin biopsies (**Fig. 3B**). In contrast, melanocytes or melanoma cells did not show remarkable PER1 or CLOCK immunopositive staining. Since PER1 and CLOCK are transcriptional regulators, the number of nuclear immunopositive cells in skin biopsies may represent the number of cells with functional molecular clocks. Immunopositive cell number was assessed per visual field without considering the total cell number within the same visual field. Therefore, our finding of 5-6 fold decrease in the more cell-rich tumorous biopsies vs. adjacent skin means even greater reduction in the proportion of functional clock cells in melanoma biopsies.

Possible Correlations of Circadian Clock Gene Expression with Histopathological Characteristics of Melanoma Malignum

In our human skin samples, there was a correlation between the expression of PER1 and the Breslow thickness and presence of ulceration of melanoma biopsies ($p=0.006$ or 0.018 , respectively). The association of PER1 immunopositivity with these histopathological features was reciprocal: e.g. thicker tumors presented greater reduction in the number of PER1 immunopositive cells in the melanoma biopsy. However, this correlation was found significant only at the protein level: the mRNA expression patterns for neither *per1* nor *per2* correlated with any histopathological features in our melanoma biopsies. In turn, the expression of *clock* showed significant correlations with certain histopathological characteristics both at the mRNA and protein levels. For *clock* mRNA levels, significant reciprocal association was found in tumorous skin biopsies with Breslow thickness ($p=0.023$). Interestingly, similar inverse correlation was seen among adjacent, non-tumorous skin samples of the paired tumor biopsies for more clinicopathological features ($p=0.018$ for Breslow thickness, $p=0.017$ for Clark level, and $p=0.005$ for ulceration). This means that in thicker tumors, clock mRNA expression in adjacent non-tumorous skin is lower if compared to biopsies adjacent to thinner tumors. Similar observations were found at the protein level: higher Clark levels ($p=0.032$), and higher mitotic rate ($p=0.046$) of the tumor was accompanied by a greater reduction in CLOCK immunopositive cell number only in adjacent, non-tumorous skin samples of the paired tumor biopsies. Furthermore, *cry1* expression showed similar reciprocal associations ($p=0.034$ for Breslow thickness, and $p=0.029$ for ulceration) in the adjacent, non-tumorous skin.

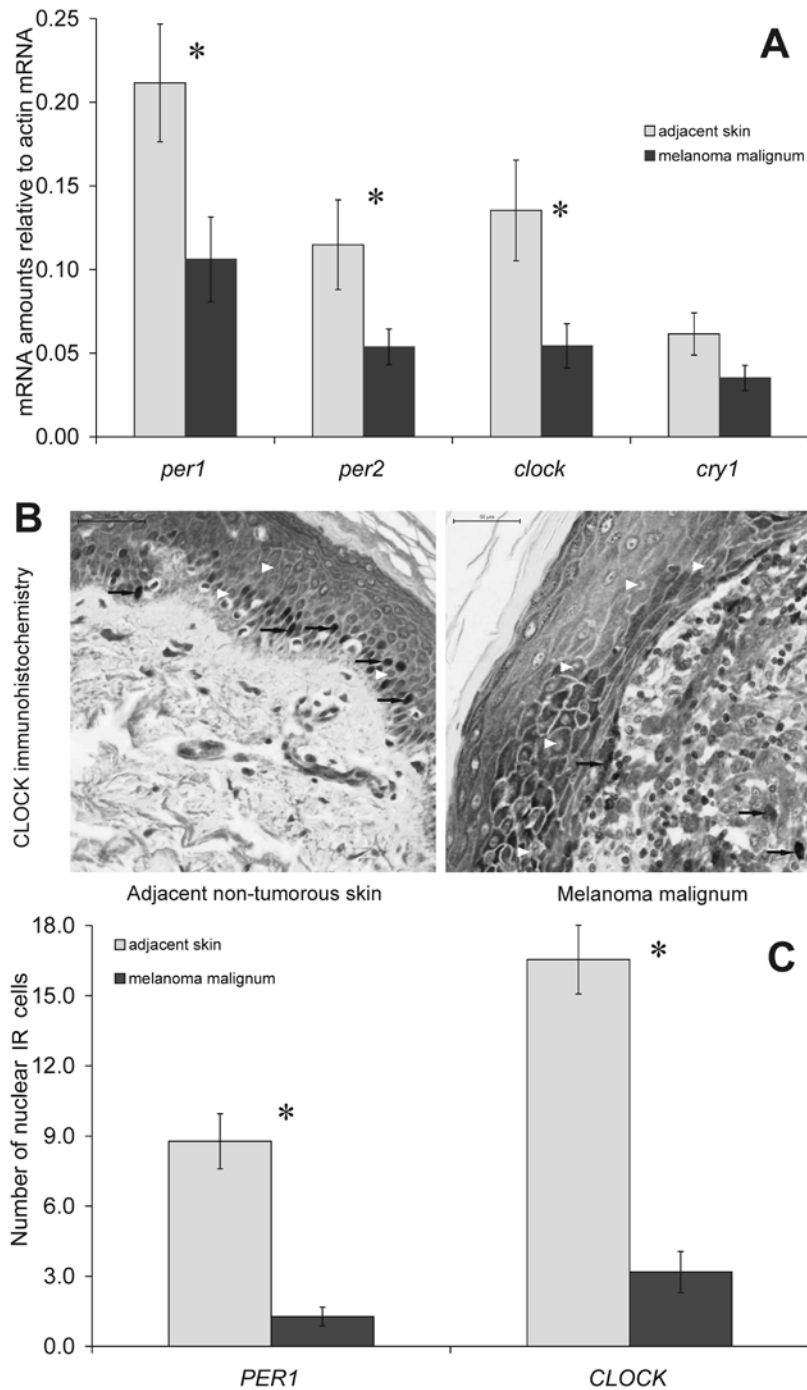


Figure 3.

A. The expression levels of circadian genes in melanoma and non-cancerous adjacent skin

B. CLOCK immunohistochemistry (black arrow: immunopositive-, white arrow : immunonegative cells)

C. Mean number of PER1 or CLOCK nuclear immunopositive cells

Expression of *Per1*, *Per2*, *Clock* and *Cry1* in Skin Biopsies from Human Naevi

Amounts of clock gene mRNAs were decreased in naevi compared to adjacent healthy skin tissues (**Fig. 4A**). In average, *per2*, *clock*, and *cry1* showed significantly decreased levels of expression (p values were 0.032, 0.008, 0.007, respectively), whilst decrease in *per1* mRNA

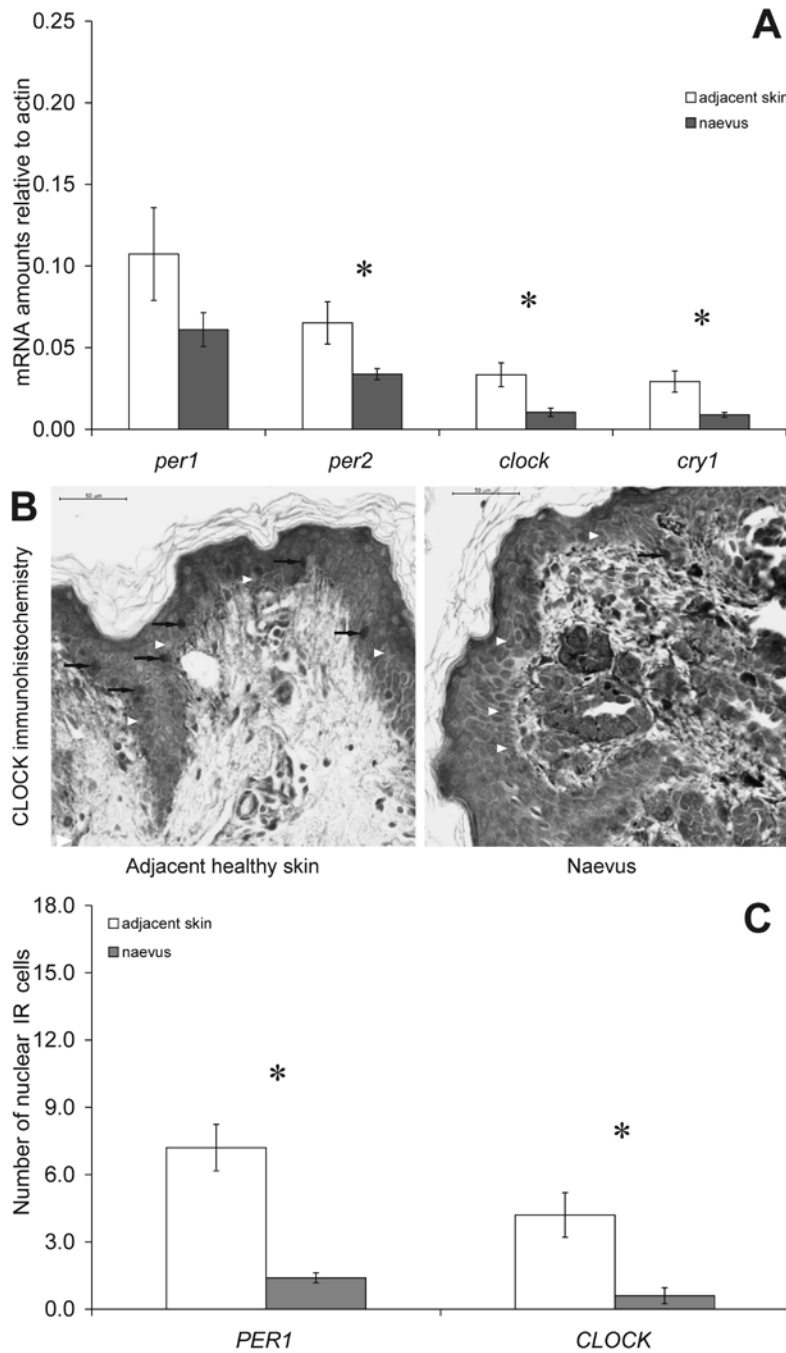


Figure 4.

A. The expression levels of clock genes in paired naevus and normal skin of volunteers

B. CLOCK immunohistochemistry (black arrow: immunopositive-, white arrow: immunonegative cells)

C. Mean number of PER1 or CLOCK nuclear immunopositive cells

expression was not significant ($p=0.145$). The number of nuclear immunopositive cells in naevi compared to adjacent healthy skin biopsies also showed a decrease (**Fig. 4C.**). Similar to that seen in our melanoma samples, melanocytes did not show remarkable PER1 or CLOCK nuclear immunopositive staining, while most of the immunopositive cells were keratinocytes. PER1 and CLOCK immunopositivity was significantly reduced in the samples

of 8 and 6 out of 10 naevus volunteers, respectively. In average, the naevus samples clearly showed an overall reduced number of immunopositive cells in PER1 or CLOCK IHC analysis ($p=0.006$ and $p=0.029$, respectively) (**Fig. 4B.**).

Comparison of clock gene expression patterns between melanoma and naevus biopsies

When comparing malignant melanoma tumors with non-malignant naevus biopsies (**Fig. 5A.**), mRNA levels from *clock* and *cry1* were seen increased in the melanoma (5.24 and 3.98 folds, with $p=0.018$ and 0.002 , respectively). On the other hand, *per1* and *per2* mRNA levels did not differ significantly ($p=0.74$ and 0.54 , respectively). Similarly, the number of CLOCK nuclear immunopositive cells in melanoma were seen increased (5.3 fold, $p=0.011$), but the number of PER1 cells did not differ significantly ($p=0.91$). Comparing the non-tumorous adjacent skin biopsies of melanoma patients with the normal healthy skin biopsies of naevus patients (**Fig. 5B.**), mRNA levels from *cry1* and *clock* again were increased in the non-tumorous biopsy of melanoma patients (2.1 and 4.0 folds, with $p=0.027$ and 0.003 , respectively). In turn, *per1* and *per2* mRNA levels did not differ significantly (p values were 0.8, and 0.9 respectively). The number of CLOCK nuclear immunopositive cells in the non-tumorous adjacent skin biopsies of melanoma patients were increased (3.94 fold, with $p<0,001$), but the number of PER1 cells did not differ significantly ($p=0.75$).

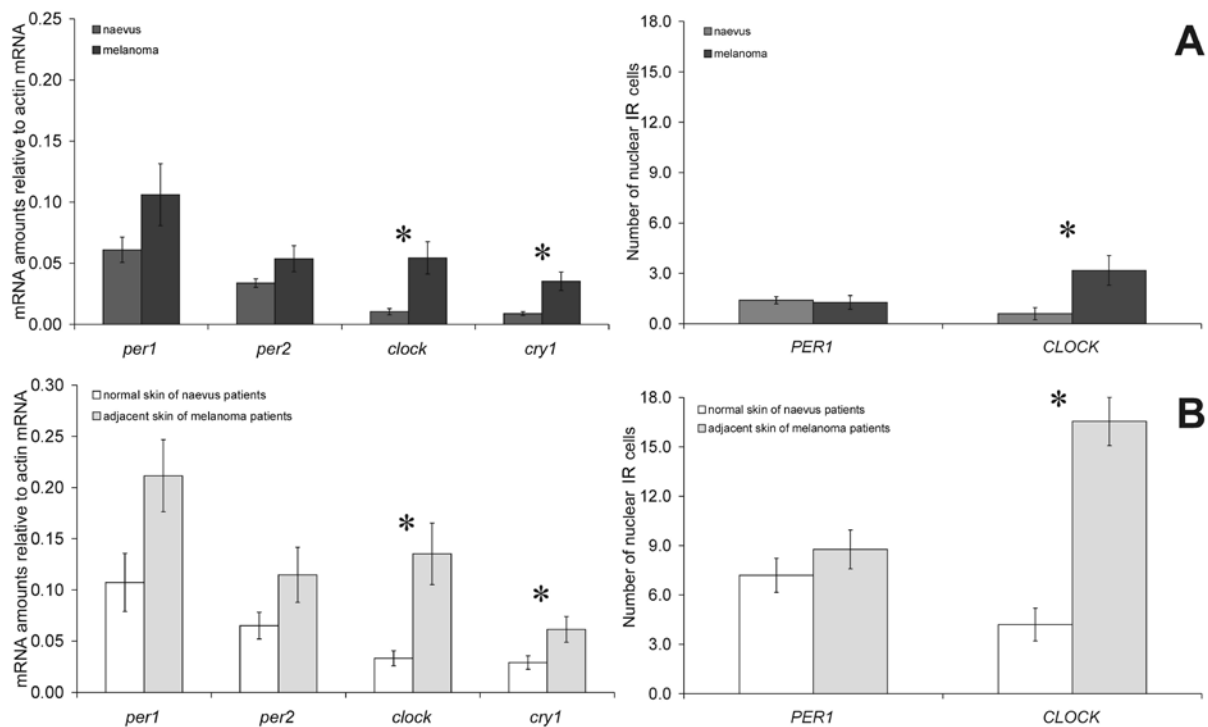


Figure 5.

- A.** Comparison of clock gene expression in tumorous samples of melanoma vs. naevus
B. Comparison of clock gene expression of adjacent non tumorous skin of melanoma patients vs. normal skin of naevus

5. DISCUSSION

Findings on the daily rhythmic nature of cell cycle control, DNA damage response and apoptotic mechanisms opened a new path in tumor biology research. Some of the components of the circadian molecular clock are directly involved in tumor suppression (*per1*, *per2*), while the roles of other clock genes in this process remain less clear (*clock*, *bmall*, *cry*). Human tumorous biopsies of various cancer types consistently show down-regulated clock gene expression as cca. 0.5 fold reduction in mRNA contents at the tissue level if compared to their adjacent non cancerous biopsies. This phenomenon not necessarily caused by malignant transformation, but could be simply due to altered cellular composition of the tumorous biopsy.

Skin is an ideal model to study the relationship of circadian timing and tumor development: (i) it contains functional peripheral clocks within different cell types of its heterogenous cellular composition; (ii) a role of the circadian system in skin tumors has been proven in animal experiment.

Having taken these into consideration we determined clock genes expression in melanoma samples at mRNA and protein levels. In concordance with several reports on other human tumor types we also have found that the expression of clock genes is significantly lower in the tumor biopsy than in the adjacent non-tumorous skin (**Fig. 3.**). The moderate decrease of clock genes expression and the lack of clear associations with the melanoma prognostic factors challenged the hypothesis that the altered cellular composition itself might be responsible for the downregulation of the clock genes. Therefore we obtained biopsies from benign melanocytic lesions and compared their clock gene expression to adjacent skin biopsies. When comparing to the paired adjacent skin, a decrease in clock gene expression was detected also in the naevus biopsies (**Fig. 4**), which finding was similar to that seen in melanoma patients (**Fig. 3**). These findings suggest that the moderate down-regulation of clock gene expression in malignant tumors may be a result of the altered cellular composition between tumor and adjacent non-tumorous tissues, irrespective to the presence of malignancy. On the other hand when we compared the data of naevus vs melanoma the induction of *clock* gene was detected both on transcriptional and translational levels in melanoma biopsies (**Fig. 5A.**). In our immunohistological study, the majority of PER1 or CLOCK nuclear immunopositive cells were mostly epidermal keratinocytes both in the tumor and the adjacent skin biopsies (**Fig. 3B and 4B**). The above mentioned results were seen not only in the tumor, but also in the adjacent non-tumorous skin when compared with the normal healthy skin biopsies of naevus patients (**Fig. 5B.**). The induction of CLOCK in both the tumor and in the

non-tumorous adjacent skin of melanoma patients suggests that malignancy-related metabolic, neuro-humoral, or immune factors target also non-melanocytic cellular compartments of the skin in these patients. Our data on clock gene expression gained from human melanoma biopsies does not answer the question whether the finding is a cause or consequence of malignant transformation. To clarify this further investigations are needed. Recently published reports attribute outstanding attention to CLOCK, that links circadian oscillations to metabolic regulations, hereby may play a part in tumor development and/or progression, which is suggested by our findings as well.

A better understanding of the relationship between circadian timing, tissue metabolism and tumor development is expected in the near future with a potential for new, more potent and individualized therapeutic strategies in oncology.

NOVEL FINDINGS

- (1) Being the first to confirm the expression of clock genes at mRNA and protein levels in human melanocytic lesions (melanoma and naevus).
- (2) No obvious correlations have been found between the decreased expression of the clock genes and the known prognostic factors of melanoma malignum.
- (3) Being the first to doubt, that only the malignant transformation can be responsible for the detected downregulation of clock genes, observed in tumorous biopsies when compared to adjacent non-tumorous samples. Hypothized and proved indirectly that the decrease of clock gene expression may be due to the altered cellular composition of the tissue samples.
- (4) According to our results, decrease in the expression of *per1* is not necessarily an indicator of malignant transformation in tumor biopsies.
- (5) We have demonstrated that the non-tumorous skin adjacent to melanoma is not healthy skin, because altered clock gene expression is detected, when compared to normal skin. The altered CLOCK expression in the skin may represent a perturbed metabolic environment which may either promote tumor formation (i.e. pre-cancerous stage) or affect tumor treatment efficiency.
- (6) Induction of CLOCK was observed both in the melanoma and in the adjacent non-tumorous skin at mRNA and protein levels. The transcriptional alterations of clock gene expression in malignant samples are rather characteristic of the non-malignant cells in the tumor and its surrounding than for the malignant cells.

ACKNOWLEDGEMENTS

I owe a debt of gratitude and appreciation to my advisors, Dr. Zita Battyani and Prof. Dr. Valér Csernus, who completely supported me throughout my PhD both in my research work and in preparing me for academic life and clinical practice.

Special thanks to Dr. Andras Nagy who helped me begin and complete my academic work. With his advice and optimism, he encouraged me to be persistent. He thought me that “you can always improve and grow”.

Thank you to Dr. György Szekeres and his colleagues in their help with imunohistochemistry and Dr. Zoltán Rékási for the opportunity to utilize his PCR machine.

I am grateful for the help of the assistants, laboratory technicians, nurses, surgical nurses, and medical students in obtaining samples.

I would like to thank the patients and study participants who participated in the examinations. Finally, but perhaps most importantly, my grateful thanks to my family who under all circumstances made it possible for me to pursue my academic activities and the preparation of the dissertation. I thank my husband for his patience, professional advice, my parents’ selfless and continuous help throughout my life, my brother for the illustration tasks, and my grandmother for the many, many hours of childcare and everyone for their encouragement.