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Rhythmic expression of circadian clock genes in human leukocytes and beard hair follicle cells

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ABSTRACT

Evaluating individual circadian rhythm traits is crucial for understanding the human biological clock system. The present study reports characterization of physiological and molecular parameters in 13 healthy male subjects under a constant routine condition, where interfering factors were kept to minimum. We measured hormonal secretion levels and examined temporal expression profiles of circadian clock genes in peripheral leukocytes and beard hair follicle cells. All 13 subjects had prominent daily rhythms in melatonin and cortisol secretion. Significant circadian rhythmicity was found for *PER1* in 9 subjects, *PER2* in 3 subjects, *PER3* in all 13 subjects, and *BMAL1* in 8 subjects in leukocytes. Additionally, significant circadian rhythmicity was found for *PER1* in 5 of 8 subjects tested, *PER2* in 2 subjects, *PER3* in 6 subjects, and *BMAL1* in 3 subjects in beard hair follicle cells. The phase of *PER1* and *PER3* rhythms in leukocytes correlated significantly with that of physiological rhythms. Our results demonstrate that leukocytes and beard hair follicle cells possess an endogenous circadian clock and suggest that *PER1* and *PER3* expression would be appropriate biomarkers and hair follicle cells could be a useful tissue source for the evaluation of biological clock traits in individuals.

1. Introduction

Circadian clocks regulate daily rhythms of physiology and behavior such as the sleep–wake cycle, body temperature, and hormonal secretion in most organisms including humans [1,2]. The circadian system is composed of three components, an input unit that transduces information for environmental changes into time signals, a self-sustained clock unit that generates autonomous circadian rhythms, and an output unit that modulates various physiological and behavioral rhythms. In mammals, the central clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus incorporates environmental information such as light–dark cycles and synchronizes phasing of clocks in peripheral cells, tissues and organs [3,4].

The molecular mechanism of the circadian system involves transcriptional and translational negative feedback loops, and post-transcriptional and post-translational modifications of multiple clock genes [5–7]. Positive transcription factors, CLOCK and BMAL1, form heterodimers and activate transcription of *Period* (*Per*) *1*, *Per2*, *Per3*, and *Cryptochrome* (*Cry*) *1*, *Cry2* by binding to E-box motifs in their promoter regions. PER and CRY proteins gradually accumulate in the cytoplasm, and casein kinase I (CKI) δ and CKI ε phosphorylate PER and CRY. PER, CRY and CKI proteins form complexes that translocate to the nucleus and interact with the CLOCK–BMAL1 heterodimers, thereby inhibiting transcription of the *Per* and *Cry* genes.

Recent studies have shown rhythmic expression of circadian clock genes in leukocytes, oral mucosa, skin and hair follicle cells under various experimental conditions [8–20]. These findings indicate that measuring clock gene expression profiles in peripheral cells may be a useful tool for the evaluation of individual circadian rhythms. Assessment of individual circadian rhythm traits is crucial for understanding the human circadian clock system [7,21,22]. Although blood has been exclusively used as a tissue source, hair might be an alternative biopsy sample because it requires only a minimally invasive procedure for sample collection.

In this study, we examined several characteristic parameters of circadian rhythm in thirteen healthy male subjects by using a 34 h measurement paradigm of continuous wakefulness and complete rest (constant routine condition), where interfering factors were strictly controlled. In addition to traditional assessment of physiological rhythms, we measured the expression level of clock genes in peripheral leukocytes and beard hair follicle cells and assessed

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the validity of using such measurements as a tool for characterizing individual circadian rhythms.

2. Methods

2.1. Subjects

Thirteen healthy, male volunteers, aged 19–23 years (mean age ± SD: 21.5 ± 1.2 years) participated in this study. Subjects had normal blood biochemistry test results, and no sleep disorders (assessed by polysomnography), psychiatric disorders (assessed by the Mini-International Neuropsychiatric Interview) or severe physical diseases. None had travelled across time zones or had been on medication in the past half year. Written informed consent was obtained from each subject.

2.2. Protocol

Constant routine protocol was performed in a sleep laboratory free of external time cues to assess subjects' circadian phases. On the day of admission, subjects were scheduled to enter the laboratory at 17:00 (shown as hh:mm), have an 800-kcal meal at 19:00, go to bed at 24:00 and sleep for 9.33 h on a bed in individual rooms. On the next day, subjects were required to lie on a reclining chair in a semi recumbent position at 11:00 and stay awake for 34 h. Blood was collected every hour using an intravenous catheter placed in a forearm vein of the subject from 12:00 to 45:00. Beard hair was collected every two hours using tweezers from 12:00 to 44:00. Water was available at all times and a 200-kcal meal was provided every two hours. Lights were kept under a low-intensity light condition (<15 lx) during the wake period and were off (0 lx) during the sleep period, and ambient temperature was maintained at 25 ± 0.5 °C in the laboratory throughout the experiment. The protocol was approved by the Institutional Ethics Committee of the National Center of Neurology and Psychiatry and was conducted in accordance with the Declaration of Helsinki.

2.3. Measurements

Blood samples (7 ml) were collected hourly in EDTA-2Na tubes (TERUMO, Tokyo, Japan). Plasma was immediately separated by centrifugation (15 min, 1600 × *g*, 4 °C) and stored at −20 °C until analysis. Plasma melatonin and cortisol concentrations were measured by radioimmunoassay using MELATONIN RIA (Buhlmann Lab AG, Basel, Switzerland) and Cortisol Kit TFB (Immunotech SAS, Marseille, France), respectively.

2.4. Gene expression analysis

Blood samples (2.5 ml) were collected hourly into PAXgene Blood RNA vacutainer tubes (PreAnalytiX, Hombrechtikon, Switzerland), mixed by inversion, incubated at room temperature for several hours, and then frozen at −20 °C until extracted using the PAXgene Blood RNA kit (QIAGEN, Hilden, Germany). Every 2 h, follicle cells were collected into the lysis buffer, mixed by vortex, and then frozen at −20 °C until extracted using the dissolution buffer (RNeasy Micro Kit; QIAGEN, Hilden, Germany). Extracted total RNA was analyzed spectrophotometrically (GE Healthcare, Buckinghamshire, UK). Synthesis of cDNA was carried out using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Forster City, CA). We performed TaqMan probe-based quantitative RT-PCR using the ABI Prism 7500 sequence detection system (Applied Biosystems, Forster City, CA) to determine the expression level of *PER1*, *PER2*, *PER3*, *BMAL1*, and *GAPDH*. Primer and probe sets used for RT-PCR assays were as follows: Hs00242988_m1 for *PER1*; Hs00256143_m1for *PER2*; Hs00213466_m1 for *PER3*; Hs00154147_m1 for *BMAL1* (TaqMan Gene Expression Assays, Applied Biosystems, Forster City, CA). The primer and probe set for *GAPDH* was designed as follows: *GAPDH* forward primer, 5′-CAAGGTCATCCATGACAACTTTG-3′; *GAPDH* TaqMan probe, 5′-FAM-ACCACAGTCCATGCCATCACTGCCA-MGB-3′; *GAPDH* reverse primer, 5′-GGGCCATCCACAGTCTTCTG-3′ [18]. Expression levels of clock genes were normalized to that of *GAPDH* at each time point.

2.5. Data analysis

We normalized the data at each time point by subtracting the population mean from the individual's raw data for level of melatonin, cortisol or relative mRNA, and then divided the difference by the standard deviation of the population (z-score). The z-score standardized data was tested for circadian rhythmicity by the fit of a 24/12 h composite cosine model using ChronoLab [23]. Cosinor *P* value for 24 h <0.05 was considered to be significant. Peak time of melatonin, cortisol, *PER1*, *PER2, PER3* and *BMAL1* rhythms for each person was designated as orthophase of the best fitting curve for individual daily profiles from those subjects who showed significant 24-h rhythms (Cosinor *P* value for 24 h <0.05). The phase for each parameter was designated as the mean peak time relative to habitual sleep onset time. Sleep onset time was determined using the data collected by sleep log and actigraphy during the seven days prior to admission. The average time of sleep onset was used as the habitual sleep onset time and set to relative time 00 h for each subject. Statistical analysis was performed using SPSS ver. 11 (SPSS Japan Inc., Tokyo, Japan). Pearson's correlation analysis was performed to ascertain whether rhythmic expression of clock genes was correlated with physiological rhythms. One-way ANOVA followed by Bonferroni *post*-test was performed to compare the phases of *PER1*, *PER2, PER3* and *BMAL1* rhythms. Unpaired *t*-test was performed to compare the phases of clock gene expression between peripheral leukocytes and beard hair follicle cells. Data are presented as mean ± standard error of the mean (SEM).

3. Results

Fig. 1 shows daily profiles of plasma melatonin and cortisol secretion. Robust circadian rhythms were seen in hormonal secretion levels from all 13 subjects (Table 1). The phase of melatonin secretion rhythm corresponded to 02:33 ± 00:35, while that of cortisol secretion rhythm corresponded to 07:40 ± 00:40.

Fig. 2 shows the average expression profiles of *PERI*, *PER2*, *PER3*, and *BMAL1* in peripheral leukocytes and beard hair follicle cells. In leukocytes, significant circadian rhythmicity was found for *PER1* in 9 subjects, *PER2* in 3 subjects, *PER3* in all 13 subjects, and *BMAL1* in 8 subjects (Table 1). The phase for daily rhythm of clock gene expression in leukocytes corresponded to 06:55 ± 00:57 for *PER1*, 05:41 ± 02:35 for *PER2*, 04:45 ± 00:59 for *PER3*, and 15:42 ± 01:58 for *BMAL1*. The phase of *PER* rhythms was significantly different from that of *BMAL1* rhythm (all $P < 0.01$). We compared the phases of clock gene expression rhythms with that of physiological rhythms for each subject (Fig. 3). The *PER1* phase showed high correlation with the melatonin phase ($R = 0.875$, $P = 0.002$) and the cortisol phase ($R = 0.817$, $P = 0.007$). The *PER3* phase also showed high correlation with the melatonin phase ($R = 0.804$, $P = 0.001$) and the cortisol phase ($R = 0.791$, $P = 0.001$).

Out of 13 subjects, 8 subjects were tested for circadian rhythmicity of clock gene expression in beard hair follicle cells. Sufficient amounts of mRNA were not obtained from the remaining 5 subjects. Significant circadian rhythmicity in beard hair follicle cells was found for *PER1* in 5 of 8 subjects tested, *PER2* in 2 sub-

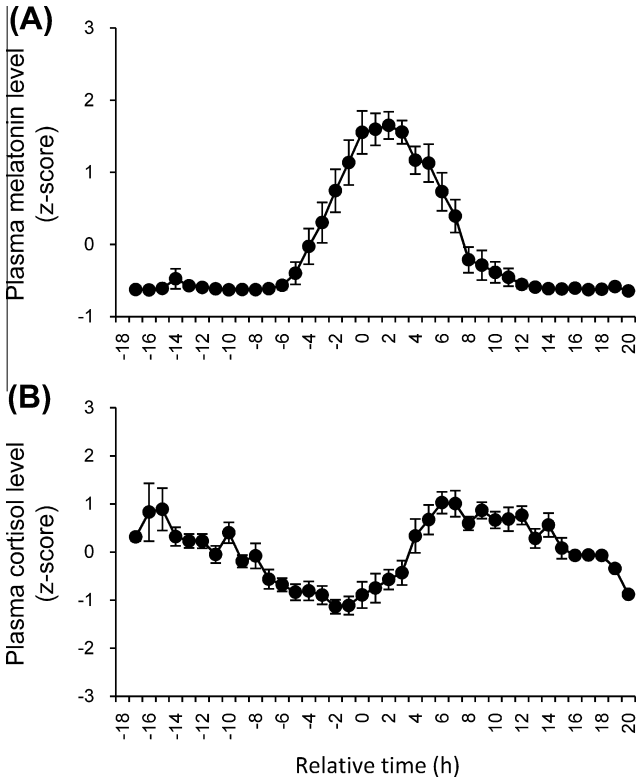

Fig. 1. Circadian rhythms of plasma hormonal secretion. Data are presented as the mean standardized z-score value ± SEM of melatonin (A) and cortisol (B) concentrations measured at 34 time points in all 13 subjects. Habitual sleep onset time is set to relative time 00 h.

Table 1
Circadian characteristics of each parameter.

Source	Parameter	Number of subjects with significant daily rhythm (Cosinor *P* value for 24 h < 0.05)	Phase (range) hh:mm ± SEM relative to habitual sleep onset time
Plasma	Melatonin	13/13 (100%)	02:33 ± 00:35 (−00:23–07:34)
	Cortisol	13/13 (100%)	07:40 ± 00:40 (02:43–12:50)
Leukocytes	*PER1*	9/13 (69.2%)	06:55 ± 00:57 (02:23–11:53)
	PER2	3/13 (23.1%)	05:41 ± 02:35 (02:48–10:50)
	PER3	13/13 (100%)	04:45 ± 00:59 (−00:07–11:41)
	BMAL1	8/13 (61.5%)	15:42 ± 01:58 (08:07–22:32)
Beard hair follicle cells	*PER1*	5/8 (62.5%)	05:50 ± 00:58 (02:08–07:45)
	PER2	2/8 (25.0%)	05:49 ± 00:13 (05:35, 06:02)
	PER3	6/8 (75.0%)	06:14 ± 00:55 (03:03–08:57)
	BMAL1	3/8 (37.5%)	18:37 ± 00:18 (18:03–19:06)

jects, *PER3* in 6 subjects, and *BMAL1* in 3 subjects (Table 1). The phase for each parameter in follicle cells corresponded to 05:50 ± 00:58 for *PER1*, 05:49 ± 00:13 for *PER2*, 06:14 ± 00:55 for *PER3*, and 18:37 ± 00:18 for *BMAL1*. As anticipated, the phase of *PER* rhythms was significantly different from that of *BMAL1* rhythm (all $P < 0.01$). However, no significant differences were observed in the phases of clock gene expression between peripheral leukocytes and beard hair follicle cells ($P > 0.05$).

4. Discussion

Daily rhythms of plasma melatonin and cortisol secretion were observed in all 13 subjects and their phases were comparable to previous studies [18,24]. In terms of experimental conditions, therefore, neither masking effects nor possible disruption due to the collection of samples at 1-h or 2-h intervals had a significant interfering effect.

Rhythmic expression of circadian clock genes was observed in peripheral leukocytes under the constant routine condition and the expression pattern of *PER1*, *PER2* and *PER3* was antiphasic to that of *BMAL1* as anticipated. These findings demonstrate that human peripheral leukocytes and hair follicle cells possess endogenous circadian properties despite the limited number of subjects assessed. This is the first study to show that hair follicle cells as well as leukocytes possess an endogenous circadian clock. Akashi et al. have developed a convenient method for examining circadian clock gene expression using collected scalp hair follicle cells [20]. Our data provide additional information and further evidence validating their method. Hair could be an ideal biopsy sample for the evaluation of individual biological clock traits by virtue of the non-invasive nature of sample collection. It would be worthwhile to increase the sample size for a thorough validation of the possibility of using hair follicle cells as a tissue source for surrogate circadian measurements.

We detected rhythmic *PER2* expression in only 3 of 13 (23.1%) subjects in leukocytes. Additionally, another group has reported that *PER2* mRNA level was rhythmic in only 8 of 24 (33%) subjects in leukocytes under a constant routine condition (with sleep deprivation) [18]. In contrast, we have previously found *PER2* rhythm in PBMCs from young subjects (62.5%) and older subjects (66.7%) under a semi-constant routine condition (without sleep deprivation) [15,19]. Other groups also have reported rhythmic *PER2* expression in PBMCs under non-sleep-deprived conditions such as simulated night shift work conditions [17] and hospitalized conditions [14]. These data imply that masking effects such as sleep and physical movement might generate rhythmic expression of *PER2*. On the contrary, all subjects rhythmically expressed *PER3* in their leukocytes. In mice, *Per3* mRNA level is not induced by light exposure in the dark phase while *Per1* and *Per2* mRNA levels are induced immediately and transiently [25]. The *Per1* gene contains a canonical cAMP responsive element (CRE) and canonical E-boxes in the promoter region [26]. The *Per2* promoter also contains a canonical CRE as well as a non-canonical E-box [27,28], while the *Per3* promoter does not have a CRE element but does contain E-boxes [27]. CRE-mediated transcription is regulated by multiple signaling pathways activated by a variety of stimuli including light exposure [29]. In contrast, E-boxes are the target site for the CLOCK–BMAL1 heterodimer, which is thought to be the key driver of circadian expression of clock genes [3,26,30–32]. It appears that a combination of regulatory elements may contribute to *PER* expression patterns and that *PER3* may have distinct drivers that maintain its rhythmic expression.

The phase of *PER1* and *PER3* rhythms correlated closely with that of melatonin and cortisol secretion rhythms, which are conventionally used as circadian phase markers. Our previous study has indicated that *PER1* phase in peripheral blood mononuclear cells (PBMCs) is correlated with melatonin phase [10]. Additionally, another group has shown that *PER3* rhythm in whole blood cells is associated with physiological rhythms [18]. Our results along with those of others strongly suggest that the phase of *PER1* and *PER3* rhythms in peripheral blood could provide an accurate measure of individual circadian rhythms, and thus could serve as a useful biomarker for the evaluation of biological clock traits in individuals.

It is well documented clinically that the risks for sleep disorders increase as a result of prolonged periods of habitual sleep timings that are incompatible with an individual's biological clock traits

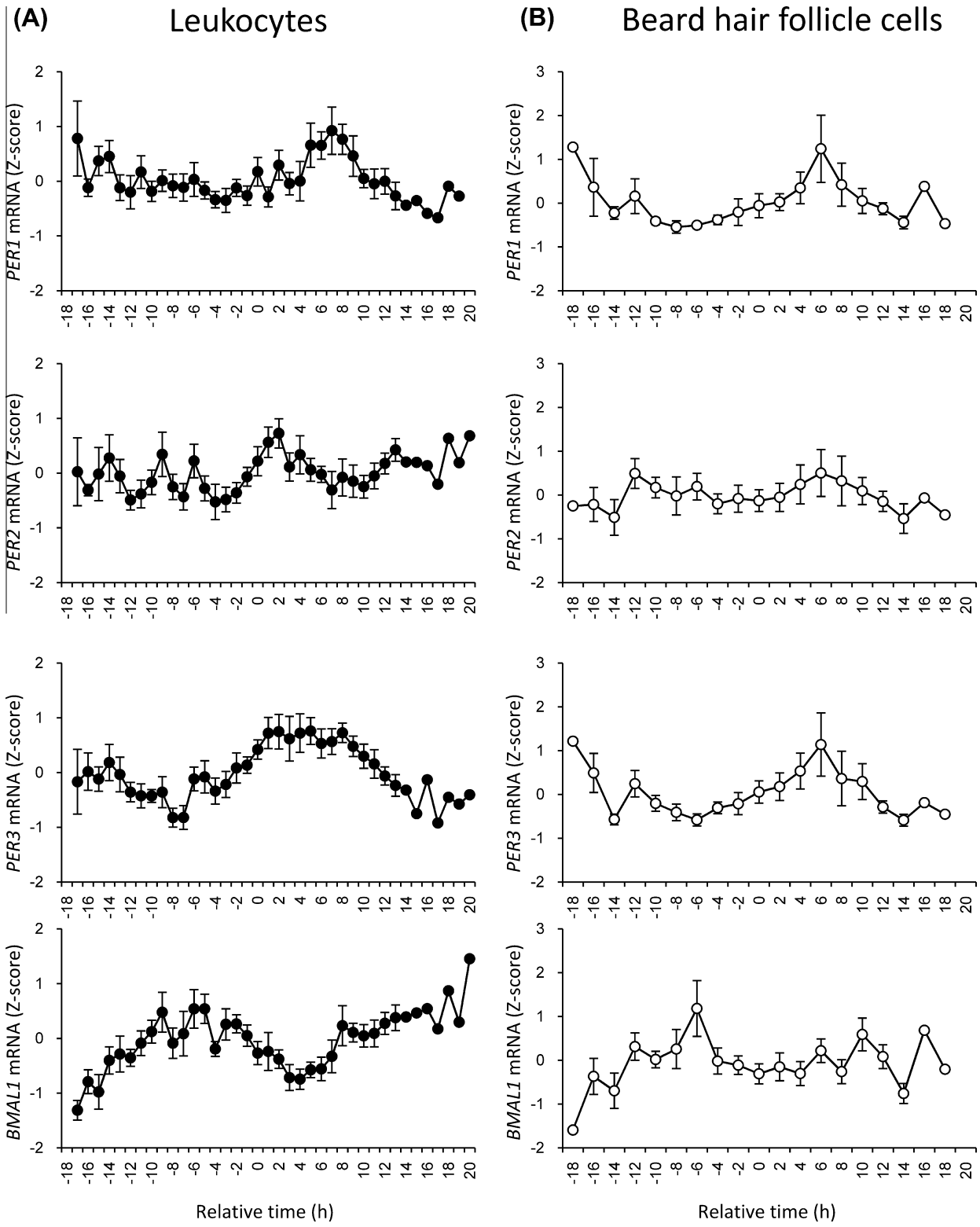

Fig. 2. Expression profiles for four circadian clock genes in leukocytes and beard hair follicle cells. Data are presented as the mean standardized z-score value ± SEM for relative *PER1*, *PER2*, *PER3*, and *BMAL1* mRNA levels in leukocytes isolated from whole blood collected at 34 time points (A) in all 13 subjects and in beard hair follicle cells collected at 17 time points (B) in 8 subjects. Habitual sleep onset time is set to relative time 00 h.

[33]. When physiological rhythms (e.g., of autonomic nervous activity and neuroendocrine secretion) and the sleep–wake cycles are misaligned with each other, a disordered state known as internal desynchronization may occur [34]. In addition, other consequences include elevated risks for mood disturbance, autonomic neurological symptoms, metabolic abnormalities and immune function impairment, as well as substantial deterioration in quality of life (QOL) [35–38].

In summary, the present study demonstrates that *PER1* and *PER3* mRNA levels show robust circadian rhythms in peripheral

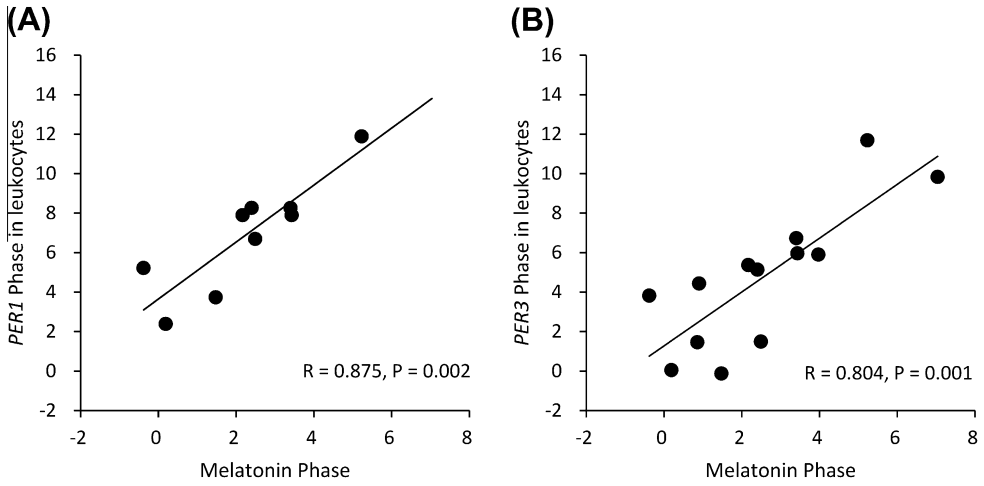

Fig. 3. Correlation between plasma melatonin secretion rhythm and *PER* rhythms in leukocytes. Strong correlation is seen between the melatonin phase and the *PER1* phase ($R = 0.875$, $P = 0.002$) (A), and the *PER3* phase ($R = 0.804$, $P = 0.001$) (B).

leukocytes and beard hair follicle cells from healthy subjects under conditions where interfering factors are kept to minimum and that *PER1* and *PER3* rhythms in leukocytes correlated significantly with plasma melatonin and cortisol secretion rhythms. These findings suggest that *PER1* and *PER3* expression in peripheral cells would be appropriate biomarkers and that hair could be a useful tissue source for the assessment of individual biological clock traits.

Declaration of interest

The authors have declared that no competing interests exist.

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