In vitro circadian period is associated with circadian/sleep preference

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Evaluation of circadian phenotypes is crucial for understanding the pathophysiology of diseases associated with disturbed biological rhythms such as circadian rhythm sleep disorders (CRSDs). We measured clock gene expression in fibroblasts from individual subjects and observed circadian rhythms in the cells (in vitro rhythms). Period length of the in vitro rhythm (in vitro period) was compared with the intrinsic circadian period, \( \tau \), measured under a forced desynchrony protocol (in vivo period) and circadian/sleep parameters evaluated by questionnaires, sleep log, and actigraphy. Although no significant correlation was observed between the in vitro and in vivo periods, the in vitro period was correlated with chronotype, habitual sleep time, and preferred sleep time. Our data demonstrate that the in vitro period is significantly correlated with circadian/sleep preference. The findings suggest that fibroblasts from individual patients can be utilized for in vitro screening of therapeutic agents to provide personalized therapeutic regimens for CRSD patients.

**Behavioral and physiological processes** such as sleep/wakefulness and hormone secretion exhibit circadian rhythms. Individual differences in daily activity/sleep time, known as the diurnal preference/chronotype, are commonly assessed using the conventional self-reported Horne-Ostberg Morningness-Eveningness Questionnaire (MEQ) and/or the recently developed online self-reported Munich ChronoType Questionnaire (MCTQ). The morning (early) chronotype manifests earlier timings for sleep and physiological rhythms such as core body temperature and melatonin secretion than the intermediate chronotype, and still earlier than the evening (late) chronotype. The various daily behavioral and physiological rhythms are regulated by a system of self-sustained clocks and are entrained to environmental cues, such as light exposure, food intake, and work schedules, enabling us to adapt to changes in the external environment. In mammals, the circadian clock system is hierarchically organized such that the central oscillator in the suprachiasmatic nuclei (SCN) of the hypothalamus integrates environmental information and synchronizes the phase of oscillators in peripheral cells, tissues, and organs. The molecular mechanism of the circadian clock system involves a complex set of transcription-translation negative feedback loops that regulate multiple clock genes including BMAL1, CLOCK, CRY, PER, ROR, and REV-ERB.

Circadian rhythm sleep disorders (CRSDs) are characterized by the inability to fall asleep and awaken at a desired time. There are several subtypes of CRSDs: advanced sleep phase type (ASPT), delayed sleep phase type (DSPPT), and free-running type (FRT). ASPT patients show extremely advanced involuntary timing of sleep and wake, DSPPT patients show significantly delayed sleep onset and wake times, and FRT patients have sleep times that occur with ~1-h delay each day and are not able to adapt to the external 24-h day. CRSDs are attributed etiologically to malfunction and/or maladaptation of the circadian clock system, and therefore evaluation of circadian phenotypes is crucial for understanding the pathophysiology of CRSDs. The intrinsic circadian period, \( \tau \) (the free-running period of circadian rhythms in the absence of external cues), is considered to be a critical factor in the pathophysiology of CRSDs. Indeed, we recently demonstrated that \( \tau \) was significantly prolonged in FRT.

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patients under a strict forced desynchrony (FD) protocol compared to healthy subjects with the intermediate chronotype. However, although the FD protocol is regarded as the most reliable and valid method for the assessment of \( \tau \) in humans, it is laborious and costly to perform in clinical settings. A more convenient evaluation of circadian phenotypes is therefore required both to reduce burden on the subjects and to increase the feasibility of examination.

To this end, Brown et al. developed a luminescence rhythm assay system using biopsy samples to evaluate an individual’s circadian phenotype. In this system, the biopsy-derived fibroblasts are transfected with a circadian reporter, the \( \text{Bmal1-luc} \) utilizing an electroporation system. After the cultured cells were synchronized by treatment with dexamethasone for 2 h, bioluminescence rhythms from the cells were continuously measured for 5 cycles. (b) \textit{In vitro} period length of \( \text{Bmal1-luc} \) rhythms in 9 intermediate type subjects (green circles) and 8 evening type subjects (blue circles). Data are presented as mean value ± standard error of the mean.

Figure 1 | An individual’s circadian rhythm \textit{in vitro} and the period length of \textit{in vitro} rhythms. (a) Representative detrended data of \( \text{Bmal1-luc} \) rhythm in cultured fibroblasts from subject I4. Primary fibroblasts were obtained from a skin biopsy sample and were transfected with the circadian reporter \( \text{Bmal1-luc} \) utilizing an electroporation system. After the cultured cells were synchronized by treatment with dexamethasone for 2 h, bioluminescence rhythms from the cells were continuously measured for 5 cycles. (b) \textit{In vitro} period length of \( \text{Bmal1-luc} \) rhythms in 9 intermediate type subjects (green circles) and 8 evening type subjects (blue circles). Data are presented as mean value ± standard error of the mean.

Results
Circadian rhythms were sustained in cultured cells for several cycles, as indicated by luminescence levels (Fig. 1a). The \textit{in vitro} period length of \( \text{Bmal1-luc} \) rhythm \((\text{in vitro period})\) varied among fibroblasts from different individuals (Fig. 1b, Table 1). The \textit{in vivo} period length of melatonin rhythm \((\text{in vivo period})\) had been determined for each subject in our previous study. The average \textit{in vitro} period was significantly shorter than the average \textit{in vivo} period in our subjects.

To address these issues, in this study we measured clock gene expression in primary fibroblasts derived from subjects’ skin biopsy samples using a non-viral luminescence assay system, and compared the period of \textit{in vitro} rhythms with \( \tau \) measured under a strict FD protocol and circadian/sleep parameters evaluated by questionnaires, sleep log, and actigraphy in a real-life setting.

Results
Table 1 | Period length of in vitro and in vivo rhythms

| Subject (number) | Age (years) | in vitro rhythm period length (h) | ±SD | in vivo rhythm period length (h) |
|------------------|-------------|----------------------------------|-----|---------------------------------|
| I1               | 19          | 22.07                            | 1.29| 23.95                           |
| I2               | 23          | 22.97                            | 1.00| 24.03                           |
| I3               | 21          | 23.30                            | 1.23| 24.17                           |
| I4               | 22          | 23.21                            | 0.45| 24.10                           |
| I5               | 22          | 23.08                            | 1.27| 24.00                           |
| I6               | 20          | 22.72                            | 0.92| 24.10                           |
| I7               | 24          | 22.97                            | 1.30| 24.26                           |
| I8               | 22          | 24.14                            | 1.02| 24.31                           |
| I9               | 39          | 23.33                            | 1.34| 24.14                           |
| mean             | 23.56       | 23.09                            | 5.98| 0.55                            |
| SD               |             |                                  | 5.98| 0.55                            |

Evening type (8)

| Subject (number) | Age (years) | in vitro rhythm period length (h) | ±SD | in vivo rhythm period length (h) |
|------------------|-------------|----------------------------------|-----|---------------------------------|
| E1               | 22          | 24.03                            | 1.49| 24.36                           |
| E2               | 22          | 22.63                            | 1.14| 24.23                           |
| E3               | 20          | 23.84                            | 1.09| 24.89                           |
| E4               | 22          | 23.21                            | 0.99| 24.68                           |
| E5               | 20          | 24.38                            | 2.20| 24.44                           |
| E6               | 23          | 24.58                            | 1.36| 23.93                           |
| E7               | 21          | 24.95                            | 1.12| 24.08                           |
| E8               | 22          | 23.36                            | 1.36| 24.14                           |
| mean             | 21.50       | 23.87                            | 1.07| 0.77                            |
| SD               |             |                                  | 4.42| 0.76                            |

Total (17)

| Subject (number) | Age (years) | in vitro rhythm period length (h) | ±SD | in vivo rhythm period length (h) |
|------------------|-------------|----------------------------------|-----|---------------------------------|
| mean             | 22.59       | 23.46                            | 4.42 | 0.76                            |

Figure 2 | Comparison of in vitro and in vivo rhythms between intermediate (I, green circles) and evening (E, blue circles) types. Dots represent the period length of the in vitro (horizontal axis) or in vivo (vertical axis) rhythm for each subject. No significant correlation was found between in vitro and in vivo periods when all subjects were examined. A longer in vitro period was observed in evening types compared to intermediate types (P = 0.028).
Additionally, there was a significant correlation between the in vitro period and habitual sleep time ($R = 0.632, P = 0.007$) (Fig. 3b). By contrast, the in vivo period was not associated with MEQ score ($R = -0.046, P = 0.860$) (Fig. 3a) or habitual sleep time ($R = -0.060, P = 0.819$) (Fig. 3b). Correlations between the in vitro or in vivo period and circadian/sleep parameters were assessed using mid-sleep timings on work days (MSW), mid-sleep timings on free days (MSF), and sleep-corrected MSF (MSFsc; another indicator of chronotype) obtained by the MCTQ. No significant correlation was found between MSW and the in vitro period ($R = 0.343, P = 0.178$) or the in vivo period ($R = -0.249, P = 0.336$) (Fig. 4a). By contrast, MSF, which represents the preferred sleep timing free of social constraints, was strongly correlated with the in vitro period ($R = 0.617, P = 0.008$) (Fig. 4b), as was MSFsc, which represents chronotype ($R = 0.592, P = 0.012$) (Fig. 4c). Evening preference was associated with a longer in vitro period. There was no association between MSF ($R = -0.037, P = 0.889$) or MSFsc ($R = -0.108, P = 0.680$) and the in vivo period (Fig. 4b and 4c).

**Discussion**

Despite the fact that only a limited number of subjects were assessed in this study, the results demonstrate that an individual’s in vitro circadian period is significantly correlated with circadian/sleep preference.

Consistent with previous reports, primary fibroblast cells derived from individuals showed rhythmic expression of the circadian reporter Bmal1-luc. However, despite Hasan et al. finding that in vitro periods were longer than in vivo periods, we observed that in vitro periods were in fact shorter. There are a number of differences in the experimental conditions between the present study and previous studies, such as the reporter constructs utilized, transfection methods, and recording media. Serum factors, pH levels and Ca$^{2+}$ concentration are known to alter the circadian characteristics of in vitro rhythms. Consequently, the differences in experimental conditions between the present and past studies might account for the differences observed in in vitro period length.

When in vitro and in vivo circadian rhythms were compared, a moderate but significant correlation between the two rhythms was observed in intermediate types, but not in evening types or in all subjects. Additionally, Hasan et al. found no correlation between in vitro and in vivo periods in their subjects. Pagani et al. reported that the in vitro period was proportional to the in vivo period in their subjects, although they did not observe a longer in vitro period in blind subjects who are known to have a significantly longer in vivo period than sighted subjects. In vivo rhythms such as core body temperature and melatonin secretion are known to be affected by the after-effects of entrainment. Long-term effects of previous long sleep-wake cycles might cause the long in vivo period in blind subjects who are known to have a significantly longer in vivo period than sighted subjects. In vivo rhythms such as core body temperature and melatonin secretion are known to be affected by the after-effects of entrainment. Long-term effects of previous long sleep-wake cycles might cause the long in vivo period in blind individuals. These data suggest that in vitro rhythms are not strongly correlated with in vivo rhythms.

The relationship between the central and peripheral oscillators has been studied by measuring luminescence rhythms in cultured SCN cells and peripheral tissues explanted from circadian reporter transgenic animals. The period and the phase of SCN rhythm are...
different from those of peripheral rhythms even under the same condition (i.e., in organotypic slice culture)\textsuperscript{31,32}. This implies that individual tissues show distinct circadian characteristics under conditions in which the tissues are dissociated. Primary fibroblasts used in this study are a group of dissociated cells established from skin biopsy samples. Unlike tissues \textit{in vivo}, cultured cells do not receive environmental information or any circadian signals from other tissues (SCN and periphery). On the contrary, almost all of the \textit{in vivo} tissues are co-regulated or are interdependent even when masking effects are minimized. It was recently reported that age-related differences are observed for numerous characteristics of behavioral and physiological rhythms but not in the molecular machinery of peripheral circadian clocks\textsuperscript{37}. These findings imply that \textit{in vitro} rhythms reflect the molecular mechanism of circadian clock components in peripheral cells, whereas \textit{in vivo} rhythms reflect the physiological mechanism of the circadian clock system of an individual.

The longer \textit{in vitro} period observed in evening types compared to intermediate types in this study is in agreement with a previous report that extreme evening types had a longer \textit{in vitro} period than extreme morning types\textsuperscript{22}. Furthermore, \textit{in vitro} period length, but not \textit{in vivo} period length, was significantly correlated with MEQ score (chronotype), habitual sleep time, MSF (preferred sleep time), and MSFsc (chronotype). Our data strongly support the notion that the period length of circadian rhythms in fibroblasts from individuals represents their circadian/sleep preference. By contrast, the \textit{in vivo} period was not correlated with any of these parameters. The \textit{in vivo} period converges to a nearly 24-h period\textsuperscript{20,33} and does not vary greatly among individuals. This characteristic of \textit{in vivo} rhythms

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**Figure 4 | Correlations between the \textit{in vitro} (circles) or \textit{in vivo} (triangles) period and (a) MSW, (b) MSF, or (c) MSFsc.** Strong correlations were observed between the \textit{in vitro} period and MSF (R = 0.617, P = 0.008) or MSFsc (R = 0.592, P = 0.012) when all subjects (9 intermediate types denoted by green circles and 8 evening types, blue circles) were examined.
might weaken the correlation between the in vivo period and circadian/sleep preference. Given that genetic factors (individual traits) have a significant effect on the determination of circadian/sleep preference, these findings suggest that the properties of in vitro rhythms might reflect individual differences in circadian clock traits better than those of in vivo rhythms. Evaluating rhythmic expression of clock genes in isolated fibroblast cells might therefore be an appropriate method to assess an individual’s circadian clock phenotype.

Hasan et al. reported that MEQ score is correlated with the in vivo period but not with the in vitro period (46), which is inconsistent with our findings. Most of their subjects were intermediate types, whereas 8 of our 17 subjects were evening types. In the present study, in vitro period length varied greatly between intermediate and evening types and a significant correlation was observed between the in vitro period and chronotype. These differences in subjects might explain the discrepancy between the two sets of results. Further validation using larger cohorts should be performed to accurately determine whether the period length of the in vitro rhythm can predict an individual’s circadian clock phenotype.

In the age of personalized medicine, one of our goals is to tailor therapies to individuals based upon their specific disorders. However, sleep disorders, like many of the conditions for which therapeutic intervention would be useful, are extremely complex genetically. Even if genomic or single nucleotide polymorphism analysis were to be performed in patients with these conditions, the data obtained would not provide sufficient information to test effective new pharmaceutical agents, let alone prescribe them for treatment.

To overcome this, effective in vitro screens to test therapeutic agents are required. To this end, we have now shown that fibroblasts in culture have circadian periods correlated with the circadian clock phenotype of the individual. Thus, we believe that these isolated fibroblasts could be utilized for in vitro screening of therapeutic agents to modify circadian disruption (e.g., altered period, phase, and amplitude of circadian rhythms) to develop personalized therapies for patients with CRSDs.

Methods

Subjects. Subjects were 17 healthy males aged 19–39 years (mean age ± standard deviation (SD), 22.6 ± 4.4 years) who participated in our previous study (47). None had sleep disorders (as assessed by clinical polysomnography and the Pittsburgh Sleep Quality Index questionnaire), psychiatric disorders (assessed by a semi-structured interview with a psychiatrist and the Center for Epidemiology Studies Depression Scale questionnaire) or severe physical diseases. None had traveled across time zones or had taken any medications over the past 6 months. MEQ score was used to determine each subject’s chronotype, where a score of 16–41 denoted evening type and that of 42–58 denoted intermediate type. Accordingly, 9 subjects were classed as intermediate type (subjects 11, 12, 13, 14, 15, 16, 17, 18, and 19) and 8 as evening type (subjects E1, E2, E3, E4, E5, E6, E7, and E8).

The protocol was approved by the Institutional Ethics Committee of the National Center of Neurology and Psychiatry, and written informed consent was obtained from all subjects.

In vivo rhythm assay. A total of 17 subjects participated in a 13-day FD protocol in a sleep laboratory free from external time cues in our previous study (47). Briefly, the FD protocol was composed of 3 experiments: 1) initial assessment of circadian phase under CR—(1st CR); 2) a 28-h sleep–wake schedule (9.33 h of sleep and 18.67 h of wakefulness) for 7 days; and 3) a second assessment of circadian phase under CR (2nd CR). Throughout the experiments, lights were maintained at a low intensity (<40 lux) during the wake period and turned off (0 lux) during the sleep period. Ambient temperature and humidity in the laboratory were maintained at 25 ± 0.5 °C and 50 ± 5% relative humidity, respectively. During the periods of CR, subjects were required to lie on a reclining chair in a semi-recumbent position and stay awake for 34 h.

Water was available at all times and a 200-cc meal was provided every 2 h. Blood samples were collected every hour using an intravenous catheter placed in a forearm vein. Plasma was immediately separated by centrifugation (15 min at 1600 × g and 4 °C) and stored at −80 °C until analysis. Concentrations of plasma melatonin were measured by radioimmunoassay. Dim light melatonin onset (DLMO) time was defined as the time when plasma melatonin concentration rose from a low background level to above 10 pg/ml (48). To calculate intrinsic circadian period, τMLMDC was defined as the period length of the 1st CR and τMLMDC was conducted at the beginning and end of the FD protocol, respectively, was divided by the number of experimental days. τMLMDC was used as the period length of in vivo rhythm (in vivo period) in this study (Table 1).

Habitual sleep time. For 7 days prior to laboratory admission, subjects maintained their daily routines and slept at a regular time every night at home under a dim light condition. Their regular sleep–wake routine was verified by sleep log and actigraphy. Wrist activity was monitored with an Actiwatch (Philips Respironics) around the non-dominant wrist. Activity data were analyzed using computer-calculated sleep-wake determinations (49). Average sleep onset and wake times during these 7 days were used as the habitual sleep onset time and wake time, respectively. Habitual sleep time was designated as the midpoint between habitual sleep onset time and wake time.

Munich chronotype questionnaire. The MCTQ was administered on the admission day to assess the subjects’ sleep onset and wake time separately on work days or free days. MSW and MSF were calculated as the midpoint between sleep onset time and wake time on work days and free days, respectively. MSWtSc was used as another indicator of chronotype.

Skin biopsy, cell culture, and in vitro rhythm assay. A skin biopsy of the dorsal region was performed using a biopsy punch (2 mm in diameter and 7 mm in length) with a plunger system (Kai Industries) on the first day of the 28-h sleep–wake schedule (i.e., Day 4 of the FD protocol). Primary fibroblast cultures, derived from the skin biopsy samples, were established by culturing in DMEM/F12 (GIBCO/Life Technologies) supplemented with 20% FBS (NICHIREI BIOSCIENCES), 1% FUNGIZONE (GIBCO, Life Technologies), 0.5 μg/ml MC-210 (DS Pharma Biomedical Co., Ltd.), and 1% penicillin/streptomycin (GIBCO/Life Technologies) at 37 °C and 5% CO2. For each measurement, 3 × 105 primary cells were transfected with 3 μg of the Bmal1-luc construct Bp527-LUC using Neon (Life Technologies) and were plated in a 35-mm dish containing DMEM/F12 supplemented with 20% FBS without penicillin/streptomycin (Day 0). After 3 days (Day 3), the medium was changed to DMEM/F12 supplemented with 5% FBS and 1% penicillin/streptomycin, and on Day 10 was changed to fresh medium (DMEM/F12 supplemented with 5% FBS and 1% penicillin/streptomycin). On Day 17, 0.1 μM dexamethasone (Sigma-Aldrich) was applied and the cells were incubated for 2 h to synchronize rhythms in the fibroblasts. Luminescence from the cells was measured for at least 5 cycles in rotation (20°C) using a luminometer (DMEM/F12 supplemented with 19.4 mM glucose (final concentration 25 mM), 10 mM HEPES (Sigma-Aldrich), 0.25% penicillin/streptomycin, and 0.1 mM beetle luciferin potassium salt (Promega), using photomultiplier tubes (Hamamatsu) in a dark box at 37 °C as previously described (50).

The data were detrended by subtracting the 24-h running average from the raw data and then smoothed with a 2-h running average using Origin7.0 (OriginLab) as previously described (51). The period length of the Bmal-luc rhythm (in vitro period) was determined by regression analysis using the second to fourth peak times of the luminescence rhythm. The in vitro period for each subject is presented as the mean of 6 to 10 independent measurements ± SD (Table 1).

Statistical analysis. Kolmogorov–Smirnov tests were performed and frequencies for the parameters tested in this study were normally distributed. Paired t-tests were used to compare in vivo and in vitro period length and unpaired t-tests were used to compare the in vivo and in vitro periods between the intermediate and evening types. Correlations were assessed by Pearson’s correlation analysis. Unpaired Student’s t-tests were used to compare the physiological in vivo period and the fibroblast in vitro period between the intermediate and evening types. P < 0.05 was considered to be statistically significant. Statistical analysis was performed using SPSS ver. 11 (SPSS Japan Inc.). Data are presented as mean ± SD.

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Author contributions

A.H., S.K. and K.M. designed research. A.H., S.K., Y.O., M.E., Y.K., Yu, M., Yo, M., KN., M.W., S.A., S.H., M.K., Y.K. and K.M. performed research. A.H., S.K. and K.M. wrote the paper.

Additional information

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