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The circadian rhythm controls telomeres and telomerase activity

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ABSTRACT

Circadian clocks are fundamental machinery in organisms ranging from archaea to humans. Disruption of the circadian system is associated with premature aging in mice, but the molecular basis underlying this phenomenon is still unclear. In this study, we found that telomerase activity exhibits endogenous circadian rhythmicity in humans and mice. Human and mouse *TERT* mRNA expression oscillates with circadian rhythms and are under the control of CLOCK–BMAL1 heterodimers. CLOCK deficiency in mice causes loss of rhythmic telomerase activities, *TERT* mRNA oscillation, and shortened telomere length. Physicians with regular work schedules have circadian oscillation of telomerase activity while emergency physicians working in shifts lose the circadian rhythms of telomerase activity. These findings identify the circadian rhythm as a mechanism underlying telomere and telomerase activity control that serve as interconnections between circadian systems and aging.

1. Introduction

Circadian rhythms exist in organisms from archaea to humans [1]. In mammals, the central clock of the hypothalamic suprachiasmatic nucleus and peripheral clocks in tissues coordinate multiple aspects of behavior and physiology [2]. The oscillatory rhythms regulate cell growth, hormonal homeostasis, electrolyte balance, energy metabolism, cardiovascular physiology, and sleep, with external environmental changes synchronizing with the rotation of the earth [3]. Disturbance of circadian rhythms often results in abnormal energy metabolism, increased carcinogenesis, cardiovascular diseases, sleep disturbances, and early aging [4]. Recent advances have supported evidence for a link between circadian rhythms and the physiology changes in aging. Flies with disturbed light/dark cycles or mutations in core circadian genes have reduced life span [5]. Mice deficient in BMAL1, CLOCK, or PER1/2 proteins develop early aging phenotypes [6]. In human, disturbances in circadian timing in shift workers is associated with an increased risk of coronary artery diseases and cancer [7].

Telomeres are DNA–protein complexes that protect chromosome ends and maintain genomic integrity. Telomeres shorten with cell division and impose a replicative limit on the growth of primary cells in culture. Telomere shortening is mainly compensated by the enzyme telomerase, which adds back telomeric DNA [8]. The regulation of telomerase activity requires integration at multiple levels and includes TERT expression control, enzyme phosphorylation, telomere complex subunit assembly, and transport [10].

The aging process in humans is associated with changes in circadian rhythm patterns [11]. Aged mice have decreased sensitivity to the effects of light entrainment and show reduced amplitude of circadian gene expression [4]. Cellular senescence impairs circadian rhythmicity, and introduction of telomerase rescues clock genes expression that has decreased due to senescence [12]. Cancer cells also have oscillatory rhythms in DNA synthesis and telomerase activity [13]. These findings suggest potentially important links between circadian rhythms and telomeres or telomerase. However, the exact mechanisms and potential interactions between circadian rhythms and telomere are still unclear.

2. Materials and methods

2.1. Cell culture and antibody

Multiple and independent mouse embryonic fibroblasts (MEFs) from wild-type and *Clock*$^{-/-}$ knockout mice were derived and assayed. To assess the oscillation of gene expression, cells were

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synchronized using serum shock. Antibodies against mTERT (Santa Cruz), Per2 (Millipore), Rev-Erbα (Cell Signaling), and Lamin A/C (Abcam) were used for Western blot.

2.2. Animals

Clock$^{-/-}$ mice on a C57BL/6J background were obtained from Dr. Reppert and Dr. Weaver (University of Massachusetts Medical School, Worcester, MA) [14]. The animal experiments were in accordance with the guidelines of Chang Gung University and Chang Gung Memorial Hospital Institutional Animal Care and Use Committee.

2.3. Telomerase assay

Telomerase extracts were prepared with CHAPS lysis buffer. The telomerase repeat amplification protocol (TRAP) assay was performed with 0.1/0.4 μg protein from the mouse liver/lung or 0.01 μg protein from human peripheral blood leukocytes. After PCR amplification and electrophoresis, gels were stained with SYBR Green nucleic acid gel stain (Molecular Probes).

2.4. Transcriptional assay

hTERT promoter regions of various lengths in pGL3-basic plasmids (Promega) were gifts from Dr. Hiroyuki Kugoh (Tottori University, Yonago, Japan). pcDNA3.1 constructs containing *mCry1* and *mCry2* were gifts from Dr. Steven M. Reppert (University of Massachusetts Medical School, MA, USA). The cells were lysed 48 h after transfection and analyzed with a dual-luciferase reporter assay system (Promega).

2.5. Chromatin immunoprecipitation (ChIP)

ChIP was performed with livers as previously described [15]. The cross-linked nuclei were immunoprecipitated using an antibody against CLOCK (Abcam) or BMAL1 (Millipore) or c-Myc (N-262, Santa Cruz). Primers for regions downstream of the TERT promoter were used as negative control. The result was normalized to input control without immunoprecipitation and reported as percent input.

2.6. Telomere Q-FISH

First-passage MEFs were incubated with 0.1 μg/mL colcemide (Gibco) overnight and then fixed in 3.7% formaldehyde in Tris-buffered saline (TBS) for metaphase spreads. Quantitative fluorescent *in situ* hybridization (Q-FISH) was performed with the telomere peptide nucleic acid (PNA) FISH kit/FITC (DakoCytomation) according to the manufacturer's protocol.

2.7. Human subjects

Participants received the Munich Chronotype Questionnaire and Pittsburgh Sleep Quality Index evaluation to exclude sleep disorders and mid-sleep on free days (MSF; the half-way point time between sleep-onset and sleep-end) earlier than 1:00 AM or later than 8:00 AM. Non-emergency physicians were from the departments of pathology, nuclear medicine, radiation oncology, and otolaryngology. Blood tests were performed at the ends of the night shift at 10:00 AM and 5:00 PM later. The clinical research studies were conducted according to the principals by the *Declaration of Helsinki*. Written informed consent was obtained from all study participants prior to enrolment. The protocol was approved by the institutional review board of Chang Gung Memorial Hospital.

2.8. Statistical analysis

Physiological and molecular parameters were analyzed with a cosinor model to determine whether there is a 24 h rhythm. A mixed model analysis of variance was used with standard least-square regression and the restricted maximum likelihood method with SPSS (IBM) and ClockLab software (Actimetrics) to determine the amplitude and phase of the sinusoidal function and to determine whether there were significant relationships. Values were expressed as mean ± standard deviation. Data were compared using Student's *t*-tests or analysis of variance (ANOVA) or Lomb–Scargle analysis, where appropriate. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Circadian oscillation of telomerase activity

To determine whether telomerase activity exhibits circadian rhythms, we assessed telomerase activity at 4-h intervals in the livers and lungs of wild-type mice. The mice were analyzed on the first day under constant darkness (DD) after entrainment for 14 d under a 12 h light/12 h dark (LD) cycle. Under these conditions, telomerase activity was rhythmic and exhibited significant circadian rhythmicity (Fig. 1A and B). The peak telomerase activity was noted at circadian time (CT) 17 and the trough at CT 1, with a peak-to-trough ratio of 2.1. Transcriptional control of telomerase reverse transcriptase (TERT) plays a crucial role in the regulation of telomerase activity [16]. It is possible that mouse *TERT* mRNA and protein also exhibit a circadian rhythm to drive the oscillation of telomerase activity. To test this possibility, we first searched the circadian expression profiles data base (CircaDB) [17]. In CircaDB, *mTERT* transcripts from mouse livers showed significant oscillations (Supplementary Fig. 1). We then examined the *mTERT*, *mPer2*, and *mRev-Erbα* mRNA expression levels in the same livers that were analyzed for telomerase activity. *mTERT* transcripts in livers and lungs showed circadian oscillation with a peak-to-trough ratio of 1.7. *mPer2* and *mRev-Erbα* transcripts have higher amplitude circadian oscillations with peak-to-trough ratios of 10.7 and 14.4, respectively. *mTERT* transcripts peaked at CT 9, *mPer2* at CT 17, and *mRev-Erbα* at CT 5 (Fig. 1C). These results indicate that telomerase activity and *mTERT* mRNA level oscillate with an endogenous circadian rhythm.

3.2. CLOCK deficiency results in decreased telomerase activity and telomere shortening

Given the existence of a circadian rhythm in both telomerase activity and *mTERT* transcription, circadian rhythm disruption may have direct effects on telomerase activity or *mTERT* transcription. We studied the oscillation of telomerase activity in CLOCK-deficient mice. CLOCK-deficient mice have reduced life span but less severe phenotypes than BMAL1-deficient mice [18]. In CLOCK-deficient mice, behavioral rhythms are preserved, while peripheral tissue rhythms are only systemically driven [19]. We analyzed telomerase activity in CLOCK-deficient and wild-type mice on the first and second days, under constant darkness (DD) after entrainment for 14 d under a 12 h light/12 h dark (LD) cycle. The telomerase activity in the livers of CLOCK-deficient mice did not show significant circadian oscillation as compared to that for the wild-type mice (Fig. 2A). The average telomerase activity was also significantly decreased in the livers of CLOCK-deficient mice (Fig. 2A). The balance between telomerase activity and incomplete DNA replication during cell division determines telomere length [16]. The fact that CLOCK-deficient mice have no telomerase