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Cell and tissue-autonomous development of the circadian clock in mouse embryos

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ABSTRACT

The emergence of the circadian rhythm is a dramatic and physiologically essential event for mammals to adapt to daily environmental cycles. It has been demonstrated that circadian rhythms develop during the embryonic stage even when the maternal central pacemaker suprachiasmatic nucleus has been disrupted. However, the mechanisms controlling development of the circadian clock are not yet fully understood. Here, we show that the circadian molecular oscillation in primary dispersed embryonic cells and explanted salivary glands obtained from *mPER2*^{Luc} mice embryos developed cell- or tissue-autonomously even in tissue culture conditions. Moreover, the circadian clock in the primary *mPER2*^{Luc} fibroblasts could be reprogrammed by the expression of the reprogramming factors. These findings suggest that mammalian circadian clock development may interact with cellular differentiation mechanisms.

1. Introduction

The circadian clock is an intrinsic time-keeping system regulating various physiological functions such as sleep/wake cycles, body temperature and metabolism [1,2]. Accumulated evidence suggests that circadian rhythm disruption is a possible risk factor for various types of health problems such as sleep disorders, metabolic diseases and cancer [3–5]; therefore, it is important to understand the physiology and pathophysiology of the mammalian circadian clock.

Circadian rhythms are mainly generated through transcriptional/translational feedback loops of clock genes. Two transcription factors, CLOCK and BMAL1, transactivate core clock genes such as *Period* (*Per1, 2, 3*), *Cryptochrome* (*Cry1, 2*) and *Rev-Erbα* via E-box enhancer elements. Expressed PER and CRY then suppress CLOCK/BMAL1 activity, which results in the cyclical activation of these clock genes [2,6]. The *Bmal1* gene also shows cyclical expression but with an anti-phasic pattern compared to E-box-driven clock genes, because REV-ERBα cyclically activates *Bmal1* transcription [7].

In rodents, embryonic circadian rhythms continue to develop during the embryonic stage even after the maternal suprachiasmatic nucleus (SCN) has been disrupted [8], although phases of fetal circadian rhythms were affected by the disruption of maternal rhythms [9,10]. In the rat, the SCN forms morphologically from embryonic day (E) 14 through E17, and circadian rhythms in clock gene expressions in the rat SCN are not yet detectable at E19 but are already present at postnatal day (P) 3 [11–13]. On the other hand, Dolatshad et al. reported that *Per2*, *Cry1*, *Bmal1*, and *Clock* mRNAs are expressed in whole mouse embryos from E10 to P1 [14]. They reported cultures for the continuous assay of bioluminescence-based reporter assay of circadian rhythms in mouse embryonic tissues at E18 [14].

Recently, using mouse embryonic stem (ES) cells, we reported that transcriptional/translational feedback loop-based circadian molecular clocks developed gradually in ES cell-derived differentiated cells after *in vitro* differentiation cultures [15]. ES cells showed no apparent circadian molecular oscillations such as those that are seen in fully differentiated mammalian cells. However, after *in vitro* differentiation culture, apparent circadian oscillations of a clock gene reporter developed [15,16]. Moreover, reprogramming differentiated and clock oscillating cells by expression of *Oct3/4*, *Sox2*, *Klf4* and *c-Myc* resulted in the loss of circadian oscillation [15]. Therefore, based on these results, we proposed a concept of cell-autonomous development of the mammalian circadian clock depending on cellular differentiation processes.

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In this study, we used *mPer2*^{Luc} knock-in reporter mouse embryos to investigate whether development of the circadian clock in mouse embryos also correlated with cellular differentiation.

2. Materials and methods

2.1. Animals

Mature female C57BL/6J Jms Slc mice were purchased from Japan SLC (Hamamatsu, Japan) and maintained in a 12:12 light/dark cycle (lights on at 08:00, lights off at 20:00). For timed pregnancy, a female mouse advancing to estrus was paired with a homozygous *mPer2*^{Luc} knock-in male (JAX® mice #006852) for one night. The day after the overnight mating was defined as E0.5.

2.2. Preparation of *mPer2*^{Luc} knock-in mouse embryonic fibroblasts

Embryos derived from *mPer2*^{Luc} knock-in mice [17,18] were collected at E10.5 or E15.5. After removal of the head and visceral tissues, the bodies were washed in fresh PBS and minced, and the isolated cells were maintained in embryonic fibroblast medium (EFM), consisting of high glucose DMEM containing 12% FBS, 1 mM sodium pyruvate (Nacalai Tesque), 0.1 mM non-essential amino acids, GlutaMax™-I (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Nacalai Tesque).

2.3. Preparation of *mPer2*^{Luc} knock-in submandibular gland organ culture

Submandibular salivary glands (SMGs) were prepared for organ culture as described previously [19]. SMGs were harvested at the midpoint of the light phase (14:00) of each embryonic stage. SMGs were removed from the embryos using a dissecting microscope. The unilateral organ was placed on a membrane filter (Nuclepore Track-Etch Membranes, 110405, Whatman®) floating in 1.2 ml of Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque,)/F-12 (11320-033, Gibco®) supplemented with 150 μg/ml of ascorbic acid (Nacalai Tesque), 50 μg/ml of transferrin (Nacalai Tesque), and 0.1 mM of luciferin (Promega). These SMGs were sealed in a 35 mm dish with a gas-permeable FEP film (25 μm thickness) and silicon grease and maintained at 36 °C in 5% CO_2 for bioluminescence recording.

2.4. Plasmids

Doxycycline-inducible PiggyBac (PB) vectors (PB-TET-L-Myc, PB-TET-OKS), PB-CAG-rtTA Adv, and pCAG-PBase were a kind gift from Dr. Yasuhide Ohinata (RIKEN Center for Developmental Biology) [20]. A PiggyBac vector with a puromycin selection marker (PB-puro, PB510B-1) was purchased from System Biosciences (CA, USA).

2.5. Induction of reprogrammed cells

The protocol used for generating induced pluripotent stem (iPS) cells from *mPer2*^{Luc} knock-in mouse embryonic fibroblast (MEF) cells at E15.5 was based on recent reports [20]. *mPer2*^{Luc} knock-in MEFs were transfected using 16.5 μL of FuGENE 6 (Promega) mixed with 1 μg of PiggyBac transposase vector (pCAG-PBase) as well as 0.5 μg of PB-puro, 2 μg of PB-TET-L-Myc, PB-TET-OKS, and 2 μg of rtTA PB vector (PB-CAG-rtTA Adv). Two weeks after transfection, the ES-like colonies were picked and cultured in embryonic stem-cell medium (ESM), which contains Glasgow minimum essential medium (G-MEM, Wako) supplemented with a 15% fetal bovine serum (FBS, Hyclone), 0.1 mM MEM non-essential amino acids (Nacalai Tesque), 0.1 mM 2-mercaptoethanol (Sigma), 1000 units/ml leukemia inhibitory factor, and 100 U/ml penicillin and 100 μg/ml streptomycin (Nacalai Tesque).

2.6. Cell culture

ES and iPS cells established in this study were cultured on a feeder layer of mitomycin C-treated primary MEFs in embryonic stem-cell medium (ESM).

2.7. In vitro differentiation

After iPS cells were trypsinized, the feeder cells were removed by incubating the cell suspension on a gelatin-coated 35-mm or 60-mm culture dish (Nunc) for 20 min at 37 °C with 5% CO_2. Embryoid bodies (EBs) were generated by harvesting 2000 cells and seeding them onto low-attachment 96-well plates (Lipidure Coat, NOF) in differentiating medium (EFM; see above) without leukemia inhibitory factor supplementation. Two days later, EBs were plated onto gelatin-coated 24-well plates and grown for several additional weeks (Fig. 1A).

2.8. Immunofluorescence

Cells plated on coverslips and cultured for 2 days in ESM were fixed with PBS containing 3.7% paraformaldehyde for 10 min at room temperature. After washing with PBS, cells were blocked with 5% skim milk and 0.1% Triton X-100 for 45 min at room temperature, and then treated with primary antibodies, anti-Nanog 1:200 (ReproCELL,), overnight at 4 °C. After being washed in PBS, the cells were incubated with the secondary antibodies Cy3-labeled anti-rabbit IgG (1:1000, Jackson). The cells were washed in PBS and mounted with a PermaFluor Mountant Medium (Thermo Electron Corporation). The cells were observed using an LSM510 confocal laser scanning microscope (Zeiss).

2.9. Real-time bioluminescence analysis

For real-time bioluminescence analysis of the cells seeded in black 24-well plates, the medium was replaced with EFM containing 0.2 mM luciferin and 10 mM HEPES, pH 7.5 without phenol red. Cells were treated with either 100 nM dexamethasone (Sigma) or 10 μM forskolin (Sigma) for synchronization. The plates were set on the turntable of an in-house fabricated real-time monitoring system.

2.10. Real-time single-cell bioluminescence imaging

For real-time bioluminescence analysis of single cells, MEFs were plated in 35-mm culture dishes. After a 2-day culture for cell attachment, the medium was replaced with EFM containing 10 mM HEPES, pH 7.5, 0.2 mM luciferin, and 100 nM dexamethasone without phenol red. The dish was set on the stage of an LV-200 microscopic image analyzer (Olympus). Time-lapse images were collected at 60 min intervals with 5 min exposure for SMG and 59 min exposure for dispersed embryonic cells.

2.11. Data analysis

Strength of rhythmicity was defined by spectral analysis (fast Fourier transform [FFT] relative power) as relative spectral power density at the peak within the range of 20–28 h [21]. For raster plots, bioluminescence intensity data were detrended by subtracting a 24-h moving average, normalizing them for amplitude, and then color coding them, with higher than average being shown in