An In Vitro ES Cell-Based Clock Recapitulation Assay Model Identifies CK2α as an Endogenous Clock Regulator

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Abstract

We previously reported emergence and disappearance of circadian molecular oscillations during differentiation of mouse embryonic stem (ES) cells and reprogramming of differentiated cells, respectively. Here we present a robust and stringent in vitro circadian clock formation assay that recapitulates in vivo circadian phenotypes. This assay system first confirmed that a mutant ES cell line lacking Casein Kinase I delta (CKIδ) induced ∼3 hours longer period-length of circadian rhythm than the wild type, which was compatible with recently reported results using CKIδ null mice. In addition, this assay system also revealed that a Casein Kinase 2 alpha subunit (CK2α) homozygous mutant ES cell line developed significantly longer (about 2.5 hours) periods of circadian clock oscillations after in vitro or in vivo differentiation. Moreover, revertant ES cell lines in which mutagenic vector sequences were deleted showed nearly wild type periods after differentiation, indicating that the abnormal circadian period of the mutant ES cell line originated from the mutation in the CK2α gene. Since CK2α deficient mice are embryonic lethal, this in vitro assay system represents the genetic evidence showing an essential role of CK2α in the mammalian circadian clock. This assay was successfully applied for the phenotype analysis of homozygous mutant ES cells, demonstrating that an ES cell-based in vitro assay is available for circadian genetic screening.

Introduction

The circadian clock is an intrinsic time-keeping system regulating various physiological functions such as sleep/awake cycle, body temperature and metabolism [1–3]. The core component is the cell-autonomous molecular oscillator comprised of transcriptional-translational feedback loops of clock genes such as Bmal1, Clock, Period (Per1, 2, 3) and Cryptochrome (Cry1, 2) [1]. Two transcription factors CLOCK and BMAL1 transactivate the Per genes, Cry genes and Rev-ERBβ via the E-box enhancer elements. Expressed PER and CRY then suppress CLOCK/BMAL1 activity, which results in the cyclic activation of these clock genes [1,4,5]. The Bmal1 gene also shows cyclic expression but an anti-phasic pattern with E-box driven clock genes because of REV-ERBβ cyclically activate the Bmal1 transcription [6]. In these circadian feedback loops, Casein Kinase I δ/ε (CKIδ/ε) have been known essential central kinases to regulate the stability of PER proteins through their phosphorylation [7–10].

It has been reported that the master pacemaker in the suprachiasmatic nucleus (SCN) develops in the late embryonic stage, and circadian rhythms subsequently appear around birth [11,12]. Recently, our studies using mouse embryonic stem (ES) cells and in vitro differentiation culture suggested cell-autonomous development of circadian molecular oscillators in mouse ES cells during differentiation [13,14]. ES cells showed no apparent molecular oscillation, in contrast to somatic cells. However, the circadian oscillation of clock gene reporters became detectable in vitro or in vivo differentiation. Moreover, reprogramming of differentiated, rhythmic cells into pluripotent stem cells resulted in the loss of circadian oscillation [13]. These results are consistent with the notion that cell-autonomous development of the mammalian circadian clock is coupled with cellular differentiation.

Genetic screening for circadian clock genes has been successfully conducted in mice using chemical mutagenesis [15,16]. Our finding of in vitro circadian clock formation through ES cell differentiation provides us with the opportunity to develop a complementary screening system in tissue culture. We recently constructed a homozygous mutant ES cell bank which facilitates phenotypic analysis of various genes in tissue culture [17]. In the present study, we established a highly consistent differentiation protocol and conducted genetic analysis of circadian rhythm using our mutant ES cells. It has been revealed that CKIδ is essential as a central kinase of the mammalian circadian
clock [7,8], and that genetic ablation of CKI\(d\) results in the lengthening of the circadian period for 2 hours in mouse embryonic fibroblasts and suprachiasmatic nucleus [18,19]. In this study, we first tested the reliability of our in vitro circadian clock formation assay to see whether the definitive features of circadian clock such as temperature compensation and genetically determined phenotypes were correctly recapitulated using wild type ES cell line and homozygous mutant ES cell line lacking CKI\(d\) expression.

In addition to CKI\(d/e\), Casein Kinase 2 (CK2) has recently also been implicated in circadian clock regulation using genome-wide RNAi screening studies [8,20]. In species other than mammals, CK2 has been revealed to play an essential role for circadian rhythm maintenance [21,22]. However, detailed genetic analysis of CK2 has been hampered in mammals by embryonic lethality in CK2 knockout mice. We therefore chose CK2\(a\) homozygous mutant ES cell line from the homozygous mutant ES cell bank [17] and investigated the effect of CK2\(a\) deficiency on circadian rhythm.

Figure 1. Establishment of in vitro circadian clock formation assay system using ES cells. (A) Scheme of the method for developing circadian oscillation in vitro via formation of embryoid bodies (EBs). EBs were generated from 2,000 ES cells and were seeded onto low-attachment 96-well plates in differentiating medium without LIF supplementation (see Methods). Two days later, EBs were plated onto gelatin-coated tissue culture 24-well plates (putting one ES onto one well) and cultured for several weeks. Subsequently, bioluminescence in each well was monitored by using PMT-based photon counting. (B) Averaged bioluminescence traces after in vitro 7, 14, 21, or 32-day differentiation of ES cells carrying Bmal1:luc reporter (left, Dexamethasone reset; right, Forskolin reset). Data detrended by subtracting a 24-h moving average are means with standard deviation \(n=24\).

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Materials and Methods

Ethics Statement
All procedures with animals were approved by Kyoto Prefectural University of Medicine Animal Care Committee.

Mutant ES Cells
Mutant ES cell lines for casein kinase I delta (abbreviated as CK1δ or CKIδ) and casein kinase 2 alpha subunit (abbreviated as CK2α or CK2A1) were generated by insertional mutagenesis with the retroviral vector as described previously [17]. The vector insertion sites are as follows (mouse genome database mm9, July 2007): CK1δ: chromosome 11, position 12,0852,242; CK2A1: chromosome 2, position 152,053,325.

Cell Culture
Wild type ES cells, genetically mutated ES cell lines (CK1δ or CK2A1), and their revertant ES cells [17] were used for in vitro differentiation. These ES cells were cultured on the feeder layer of mitomycin C-treated primary mouse embryonic fibroblasts in ES cell medium (ESM), which contains 15% fetal bovine serum (FBS, Hyclone), 0.1 mM nonessential amino acids (Nacalai Tesque), 0.1 mM 2-mercaptoethanol (Sigma), 1,000 units/mL of leukemia inhibitory factor (LIF), and 100 units/mL of penicillin–streptomycin (Nacalai Tesque).

To establish ES cells stably expressing Bmal1:luc reporter, 3 μg of Bmal1:Luc-pT2A plasmid with Zeocin™ selection marker [13] and 1 μg of a Tol2 transposase expression vector (pCAGGS-TP) [23] were diluted in 35 μL of ESM and 12 μL of Fugene 6 transfection reagent (Promega) and mixed well. After a 15-min incubation at room temperature, the mixture was added to 2.5×10^5 ES cells. The cells were selected with 100 μg/mL Zeocin™ (Invitrogen).

In vitro Differentiation
After ES cells were trypsinized, feeder cells were removed by incubating the cell suspension on a gelatin-coated 35-mm or 60-mm culture dish for 20 min at 37°C with 5% CO2. Embryoid bodies (EBs) were generated by harvesting the 2,000 cells and seeding them onto low-attachment 96-well plates (Lipidure Coat, NOF) in differentiating medium without LIF supplementation. Embryoid bodies (EBs) were generated by harvesting the 2,000 cells and seeding them onto low-attachment 96-well plates (Lipidure Coat, NOF) in differentiating medium without LIF supplementation. After 15 minutes of incubation at room temperature, the mixture was added to 2.5×10^3 ES cells. The cells were selected with 100 μg/mL Zeocin™ (Invitrogen).

Preparation of MEFs Derived from Chimeric Embryo
Chimeric embryos were generated from homozygous CK1δ or CK2A1 mutant ES cell lines and their parental (wild type) ES cell line by injection into C57BL/6 x DBA/2 F1 hybrid blastocysts. Chimeric embryos were collected at E13.5. After removal of the heads and visceral tissues, the remaining bodies were washed in fresh PBS and minced and the isolated cells were maintained in EFM.

Real-time Bioluminescence Analysis
For real-time bioluminescence analysis of the cells seeded in 24-well black plates, the medium was replaced with EFM containing 0.2 mM luciferin (Promega) and 10 mM HEPES without phenol red. Synchronization was performed using 100 nM of dexamethasone and 10 μM of forskolin for 1 hour. The plates were set on the turntable of house-made 24-PMT head type real-time monitoring equipment [24]. The bioluminescence from each well was counted for one minute in every 20 minutes.

Data Analysis
Period lengths of bioluminescence rhythms were estimated by RAP software (CHURITSU, Nagoya, Japan) using the cosinor method and based on Fourier analysis, specific for circadian rhythms [25]. Strength of rhythmicity was defined by spectral analysis (FFT relative power) as the relative spectral power density at the peak within the range of 20–28 hr [26]. The FFT analysis was applied to the whole data for each cell. For rater plots, bioluminescence-intensity data were detrended by subtracting a 24-h moving average, normalized for amplitude, and then color coded with red (higher than average) and green (lower than average). Plots were constructed using TreeView [27].

Statistical Analysis
Statistical differences were evaluated using one-way ANOVA followed by a Bonferroni post hoc test. All statistics were calculated using GraphPad Prism version 5.0 software.

Isolation of Revertant Clones
Revertant clones were isolated by transferring homozygous clones with a FLPo expression vector followed by PCR screening for recombination events as described previously [17]. The following PCR primers at the flanking regions of the vector insertion sites were used:

- Casein kinase 1δ: 5’-tgct gga ggt ggtag gga cag tga-3’ and 5’-ggg gat ggc cgg gaa cag gta-3’
- Casein kinase 2α: 5’-ggag atg tag tag aag gaa ggt tgc-3’ and 5’-cct gtc acc ctt cca cta ttc ttc-3’

Quantitative RT-PCR
MEF feeder cells were removed by plating the culture on a gelatin-coated dish for 20 min and transferring unattached ES cells onto a fresh dish. ES cells were harvested in the Isogen reagent (Nippon Gene) and the total RNA was extracted according to the manufacturer’s instructions. Power SYBR Green PCR Master Mix (Applied Biosystems) was used for real time PCR. Transcription levels were determined in triplicate reactions after normalization to 18S ribosomal RNA. Quantitative RT-PCR analysis was performed with a StepOnePlus real-time PCR system (Applied Biosystems). The amplification protocol comprised an initial incubation at 95°C for 2 min and 40 cycles of 95°C for 30 s and 60°C for 30 s, followed by a dissociation-curve analysis to confirm specificity. Primer sequences are shown below:

- Casein kinase 1δ Forward 5’-atc gcc aag ggt gct cct-3’
- Casein kinase 1δ Reverse 5’-cca cga ggg gct ttc-3’
- Casein kinase 2α Forward 5’-tca gca ggc cca ata tga-3’
- Casein kinase 2α Reverse 5’-acc tct gtt cag gca tca-3’

Results

In vitro ES Cell-based Circadian Clock Formation Assay
To evaluate the effect of the mutations on the in vitro development of the circadian clock in ES cells, we improved our ES cell differentiation protocol and established a method for robust, reproducible and stringent circadian clock formation. Briefly, we first cultured dissociated 2,000 ES cells for two days in round-bottom low-attachment 96-well plates to allow formation of...
the embryoid body (EB). We subsequently transferred one EB into one well of 24-well plates for differentiation (Figure 1A). To monitor development of circadian oscillation, we used a wild-type (WT) ES cell line stably transfected with a Bmal1::luc reporter. Whereas no circadian oscillation of Bmal1::luc reporter bioluminescence was detected even after Dexamethasone (Dex) or Forskolin (FSK) synchronization stimuli in an undifferentiated state, weak circadian bioluminescence first appeared in cultures after 14 days but rapidly dampened (Figure 1B). The bioluminescence oscillation become more robust on day 21 and reached maximum amplitude on day 32 (Figure 1B). In addition, all examined samples represented robust circadian clock oscillation and stringent reproducibility for quantitative analysis (Figure S1). Moreover, the induced rhythms showed temperature compensation (Figure S2), indicating the canonical biological nature of the circadian clock.

Casein Kinase 1δ and Casein Kinase 2 Homozygous Mutant ES Cells

We recently constructed a homozygous mouse mutant ES cell bank using promoter trap vectors for insertional mutagenesis [17]. Currently, the bank has around 200 homozygous mutant ES cell lines and 2,000 heterozygous mutant ES cell lines. Database search of the mutant bank for the circadian period mutant identified two homozygous mutant ES cell lines, harboring mutation in CK1δ and CK2α respectively. We confirmed that homozygous mutation abolished expression of the CK1δ and CK2α gene (Figure 2A and D). As a control, we obtained revertant ES cell lines which regained CK1δ and CK2α expression respectively (Figure 2B–D) [17].

Evaluation of Developed Circadian Clock Rhythmicity from Mutant ES Cell Lines

Using this in vitro differentiation culture method, CK1δ and CK2α homozygous mutant ES cells were differentiated in vitro and Bmal1::luc bioluminescence oscillation was observed. Similar to the WT ES cells (Figure 3A left panels), CK1δ and CK2α mutant ES cells developed circadian oscillation in a differentiation culture (Figure 3A middle and right panels). Heat map plots (Figure S3) and quantitative Fast Fourier transformation (FFT) - relative power analysis (Figure 3B left panel) also indicated that circadian rhythmicity and amplitude developed progressively and reached the highest levels of power at around day 28 during the ES cell differentiation in vitro. These results suggest quantitative analysis of circadian clock formation would be possible after 28 days of differentiation.

We next conducted in-depth analyses of the CK1δ and CK2α homozygous mutations on cellular circadian rhythmicity. After 28 days in the differentiation culture, bioluminescence monitoring was performed for five days. The results revealed that the CK1δ and CK2α deficient cells exhibited significantly lengthened circadian periods compared with WT and revertant cells (Figure 4A and D). In addition, the period distribution of induced circadian clocks in CK1δ and CK2α mutants showed a slightly wider range than WT and revertant cells (Figure 4B and E). The average period length of CK1δ and CK2α mutant cells were about 3.0 and 2.5 hours longer than that of WT, respectively (Figure 4C and F). In contrast, revertant lines of both mutants showed WT-like period lengths (Figure 4G and F).

Genotype Dependent Effect on Circadian Period-length Observed in Mutant ES Cell-derived Embryonic Fibroblasts

To investigate whether the abnormal period-length observed in in vitro differentiation culture of mutant ES cells recapitulates the characters of circadian clock developed in vivo, we generated chimeric mice by injecting WT and mutant ES cells into BDF1 blastocysts. Since CK1δ knock-out mice were perinatal lethal and CK2α knock-out mice were embryonic lethal, we prepared MEFs from E13.5 chimera embryos instead of mice (Figure 5A). In these MEFs, we were able to specifically monitor bioluminescence originated from ES cell lines, because host embryos are incapable of expressing the bioluminescence marker. In addition, it has been revealed that fibroblast oscillators are not influenced by the circadian properties of neighboring cells [28]. Therefore we analyzed the bioluminescence oscillation from the mixture of MEFs composed of host-derived and ES cell-derived MEFs after three passages from embryo dissociation. PMT-based bioluminescence monitoring revealed that both CK1δ and CK2α mutant ES cell-derived MEFs displayed lengthened periods compared with WT ES cell-derived MEFs (Figure 5B). Quantitative analysis confirmed significantly longer periods in CK1δ and CK2α mutant MEFs (Figure 5C). CK1δ mutant MEFs showed 24.3 hour period, nearly two hours longer than WT ES MEFs (Figure 5D upper and middle panels). On the other hand, CK2α mutant MEFs showed divergent period distribution (Figure 5D lower panel). Since this CK2α mutant ES cell line abolished its gene expression, the observed phenotypes such as longer and variable periods may be characteristic of CK2α deficient cells. The reason for the divergent period-length of these cell was not uncertain; the loss of CK2α may have affected the circadian clock development in chimeric mice embryos with some different mechanisms from CK1δ. To our knowledge, this is the first direct genetic evidence showing the effect of CK2α deficiency on circadian clocks in mammalian peripheral cells.

Discussion

It has been revealed that CK1δ plays a distinct role in mammalian circadian clock as a central kinase phosphorylating clock proteins [7–9]. In this study, the in vitro circadian clock formation assay revealed that CK1δ deficient ES cells developed circadian clock oscillation with a ~3 hours longer period-length than WT, and these results are consistent with previously reported circadian phenotypes in MEFs and SCN from CK1δ knock-out mice [8,18,19]. Moreover, WT and revertant ES cells with normal CK1δ gene expression exhibited comparable circadian periods (see Figure 4A, B and C), suggesting in vitro clock formation assay in ES cells faithfully reproduce the genetically determined circadian rhythms in mammals. In addition, the developed circadian rhythm from ES cells after in vitro differentiation culture exhibited temperature compensation (see Figure S2). These findings revealed that the in vitro circadian clock formation assay using ES cells exactly recapitulated the circadian clock phenotype (at least in cellular or tissue level) before making mice.

We also demonstrated that CK2α deficient ES cells developed at an approximately 2.5 hours longer period-length. The role of CK2α in circadian oscillation has been implicated from RNAi-mediated knock-down and/or chemical inhibition of CK2 [20,29–31]. However, off-target effects cannot in general be excluded in RNAi and a chemical inhibitor. Although off-target effects could also accompany gene trap approach, isolation and characterization of revertant (Figure 2 and 4) would help evaluate this possibility. Furthermore, the gene knockout study of CK2α has
Figure 2. Characterization of CK1δ and CK2α homozygous mutant ES cell clones and their revertant clones. (A) Design of the gene trap vector and its insertion sites in CK1δ and CK2α homozygous mutant ES cell clones. SA, splice acceptor; hyg, hygromycin-resistance gene; pA, polyadenylation signal; Pr, phosphoglycerate kinase-1 promoter; N, neomycin-resistance gene; P, fusion gene comprised of the puromycin-resistance gene and the herpes simplex virus thymidine kinase gene; LTR, long terminal repeat. Horizontal arrows below the gene trap vector indicate ES Cell-Based In Vitro Clock Recapitulation Assay.
orientation of the N and P drug resistance genes. (B) Schematic representation of the removal of mutagenic vector sequence after FLPo/FRT recombination. Recombination events were identified by PCR primers (p1 and p2) at the flanking region of the vector insertion sites. Removal of vector sequences regenerate wild type transcripts in the revertant allele. Note that the size of the gene trap vector, exons, introns are not to scale. E, exon. (C) PCR screening for FLPo/FRT recombination events. Note that PCR product was not detected in non-recombinant clones because of the large intervening vector sequence between primers. M, 100-bp DNA ladder. (D) Relative expression level of CK1δ and CK2α mRNA in wild type, mutant and revertant ES cells. Error bars show SEM (n = 3).
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Figure 3. Development of mammalian circadian rhythm by using homozygous mutant ES cells. (A) Averaged bioluminescence traces after in vitro 7, 15, 24, or 28-day differentiation of ES cells carrying Bmal1::Luc reporter (left, wild type ES cells (WT); middle, CK1δ mutant ES cells; right, CK2α mutant ES cells). Data detrended by subtracting a 24-h moving average are means with standard deviation (n = 24). (B) FFT spectral power analysis of bioluminescences of in vitro differentiated ES cells (7, 15, 24, or 28-day). Bars indicate mean values (n = 24). One circle represented in vitro differentiated ES cells from a single EB.
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been hampered due to embryonic lethality. Our approach of in vitro ES cell differentiation circumvents the problem of embryonic lethality and presents the first unequivocal evidence showing CK2α deficiency lengthens the period in mammalian cells including MEFs, establishing CK2α as an essential mammalian clock gene.

It should be noted that ES cell-based assay does not replace other assay systems such as RNAi, chemical library screening and knock-out mouse study. We rather consider that these assay systems are complementary to each other. RNAi and chemical library screening would be appropriate to study the effect of acute knock-down of target genes in a high-throughput manner. Some of the circadian phenotypes would be revealed only in whole animal studies using knock-out mice. In contrast, ES cell-based assays would reproduce developmental process to generate circadian clock in tissue culture and allow for in-depth analysis of circadian clock formation in a time-dependent manner. The ES cell-based phenotype assay would provide an alternative approach to study gene functions in vitro.

**Conclusion**

Taken together, our results suggest that the ES cell-based in vitro circadian clock recapitulation assay is a powerful tool to evaluate genetic effects, especially when gene mutation causes embryonic lethality. Using this assay, we revealed that CK2 is an essential kinase to maintain the intact circadian period-length. Furthermore, this assay can also be utilized for ES cells-based circadian genetic screening complementary to an RNAi screening.

**Figure 4.** In vitro differentiated cells from CK1δ mutant and CK2α mutant ES cells show a longer period-length of the circadian clock than cells differentiated from wild type ES cells. After 28-day differentiation, the bioluminescence intensity in each well was monitored. (A, D) Averaged bioluminescence traces after in vitro 28-day differentiated CK1δ (A) or CK2α (D) mutant/revertant ES cells (gray, WT; red, homozygous mutant; blue, revertant). Data detrended by subtracting a 24-h moving average are means (n = 24). (B, C, E, F) Distributions and bar graphs of the period lengths of bioluminescence traces in each well. Error bars are SD. Statistical differences were evaluated using one-way ANOVA followed by Bonferroni post hoc test. doi:10.1371/journal.pone.0067241.g004
Figure 5. CK1δ and CK2α mutant MEFs developed in vivo show a longer period-length of circadian clock oscillation than wild type ES cell-derived MEFs. (A) Preparation of mouse embryonic fibroblasts (MEFs) from E13.5 chimera embryos. MEFs were maintained in EFM and their bioluminescence was monitored. Only MEFs derived from injected ES cells, not the host blastocyst-derived MEFs, contain Bmal1:luc reporter and produce bioluminescence. (B) Averaged bioluminescence traces of MEFs from CK1δ and CK2α mutant chimeric mice (gray, WT; red, CK1δ mutant MEFs; blue, CK2α mutant MEFs). Data detrended by subtracting a 24-h moving average are means (n = 24). (C, D) Distributions and bar graphs of the period lengths of bioluminescences in MEFs from CK1δ and CK2α mutant chimeric mice. Arrowheads show the mean. Error bars are SD. Statistical differences were evaluated using one-way ANOVA followed by Bonferroni post hoc test. ***P < 0.001.

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**Supporting Information**

**Figure S1** The reproducibility of the development of circadian rhythms. Representative raw bioluminescence traces of in vitro 28-day differentiated wild type ES cells.

**Figure S2** Temperature compensation of the period length from wild type ES cells carrying the Bmal1:Luc reporter after in vitro 28-day differentiation. The graph indicates the mean ± SD. The lines indicate estimation from the equation $y = 14.92x + 0.21x$ (peak) or $y = 16.74 + 0.15x$ (trough). The Q10 values between 27°C and 37°C calculated from the equation are 0.907 (peak) or 0.932 (trough).

**Figure S3** Heat map plots of bioluminescence intensity of in vitro differentiated Bmal1:Luc ES cells. Each horizontal line represents ES cells from a single EB differentiated in vitro for 7, 15, 24 and 28 days. Values above and below the mean are shown in red and green, respectively.

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**Author Contributions**

Conceived and designed the experiments: KY. Performed the experiments: YU YM JT YT KH KY. Contributed reagents/materials/analysis tools: KH. Wrote the paper: KY KH.

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