Human Circadian Molecular Oscillation Development Using Induced Pluripotent Stem Cells

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Abstract The mammalian circadian clock, which coordinates various physiological functions, develops gradually during ontogeny. Recently, we have reported the posttranscriptional suppression of CLOCK protein expression as a key mechanism of the emergence of the circadian clock during mouse development. However, whether a common mechanism regulates the development of the human circadian clock remains unclear. In the present study, we show that human induced pluripotent stem cells (iPSCs) have no discernible circadian molecular oscillation. In addition, in vitro differentiation culture of human iPSCs required a longer duration than that required in mouse for the emergence of circadian oscillations. The expression of CLOCK protein in undifferentiated human iPSCs was posttranscriptionally suppressed despite the expression of *CLOCK* mRNA, which is consistent with our previous observations in mouse embryonic stem cells, iPSCs, and early mouse embryos. These results suggest that CLOCK protein expressions could be posttranscriptionally suppressed in the early developmental stage not only in mice but also in humans.

Keywords circadian clock, human iPSC, CLOCK, posttranscriptional regulation, cellular differentiation

Circadian clock ticking at a period of approximately 24 h is a universal biological function in almost all organisms on Earth. In mammals, most tissues and somatic cells operate under a circadian clock (Yamazaki et al., 2000; Yoo et al., 2004), and the center of the mammalian circadian clock lies in the suprachiasmatic nucleus in the hypothalamus. Mammalian circadian clocks confer ~24-h rhythms on various physiological functions, such as the sleep-wake cycle, energy metabolism, autonomic nervous system activity, cardiovascular system, and endocrine activity (Lowrey and Takahashi, 2011; Bass, 2012; Masri and Sassone-Corsi, 2013), by which various physiological functions are permitted to adapt in advance to environmental changes based on the rotation of the earth on its axis.

The circadian molecular oscillations in gene expression are generated via transcriptional/translational feedback loops by a set of clock genes. The heterodimers of 2 essential transcriptional factors, CLOCK and BMAL1, transactivate the expression of several core circadian clock genes, including *Period*

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(*Per1, 2, 3*), *Cryptochrome* (*Cry1, 2*), and *Rev-Erbα* via E-box elements. PER and CRY repress the transactivation of gene expression by binding to CLOCK-BMAL1 heterodimers, whereas REV-ERBα plays a negative regulation role in the gene expression of *Bmal1* via the RORE enhancer elements (Preitner et al., 2002; Hogenesch and Ueda, 2011; Ukai-Tadenuma et al., 2011; Takahashi, 2016).

Although the circadian clock is present in almost all cells of the whole body as well as in cultured cell lines (Balsalobre et al., 1998; Yagita et al., 2001), zygotes, early embryos, and germline cells in mammals have no circadian oscillations (Alvarez et al., 2003; Morse et al., 2003; Amano et al., 2009). In recent decades, it has been revealed that the circadian rhythms develop gradually during ontogeny (Reppert and Schwartz, 1986; Davis and Gorski, 1988; Jud and Albrecht, 2006; Carmona-Alcocer et al., 2018). More recently, it has been shown that murine pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent cells (iPSCs), also have no discernible circadian transcriptional rhythm of clock genes (Kowalska et al., 2010; Yagita et al., 2010; Paulose et al., 2012). However, in vitro differentiated mouse ESCs and iPSCs develop robust circadian oscillation gradually and cell-autonomously during cellular differentiation (Kowalska et al., 2010; Yagita et al., 2010), and reprogramming the differentiated cells into iPSCs made it disappear (Yagita et al., 2010). Moreover, misregulation of cellular differentiation resulted in the failed development of circadian molecular oscillation, suggesting that the development of circadian molecular oscillation is closely correlated with the cellular differentiation process (Umemura et al., 2014). Recently, the molecular mechanisms that regulate the emergence of the circadian clock in mouse ESCs were suggested to be common in mouse ontogeny (Umemura et al., 2017). After cell lineage determination, posttranscriptional mechanisms control the expression of CLOCK protein in both mouse fetal hearts and in in vitro differentiating mouse ESCs, contributing to the emergence of the circadian clock in mammalian cells (Umemura et al., 2017). Although it has recently been reported that human ESCs have no circadian oscillation (Dierickx et al., 2017), it remains unknown whether the same molecular mechanisms suppress circadian oscillation in human development.

In the present study, we investigated the molecular mechanisms that suppress circadian oscillation by using human iPSCs. First, we demonstrated that human iPSCs have no discernible circadian molecular oscillation. Subsequently, we established in vitro differentiation methods using human iPSCs and the circadian oscillations emerged following long-term in vitro differentiation cultures. As expected, similar to the cases of mouse ESCs, iPSCs, and early embryos (Umemura et al., 2017), the expression of CLOCK protein in undifferentiated human iPSCs was suppressed despite the expression of *CLOCK* mRNA. In addition, the upregulation of CLOCK protein expression was concomitant with the emergence of robust circadian oscillation. The findings suggest that at least one of the mechanisms of circadian oscillation development in both mice and humans is regulated by the posttranscriptional suppression of CLOCK protein.

MATERIALS AND METHODS

Plasmids

For construction of the circadian oscillation reporter, a total of 0.5 kb of the 5′-flanking region of the mouse *Bmal1 gene* cloned from *Bmal1:luc*-pT2A (Yagita et al., 2010) or a total of 0.4 kb of mouse *Per2* genes cloned from *Per2:luc*-pT2A (Umemura et al., 2017) was inserted into the BglII/ClaI site of a *piggyBac* (PB) transposon vector with a puromycin selection marker (*Bmal1:luc*-pPB or *Per2:luc*-pPB), which was made of a PB510B-1 vector (System Biosciences, Palo Alto, CA) by inserting a pair of oligo with BglII and ClaI sites (5′-CATGGAGATCTATCGATG-3′, 5′-AATTCATCGATAGATCTC-3′) into an NcoI/Eco RI site.

Cell Culture and Transfection

Human iPSCs (253G) purchased from RIKEN BioResource Research Center (Ibaraki, Japan) were maintained on mitomycin C–treated SNL 76/7 feeder cells (DS Pharma Biomedical, Osaka, Japan) in Primate ESC medium (ReproCELL, Kanagawa, Japan) comprising 4 ng/mL recombinant basic fibroblast growth factor (Wako, Osaka, Japan). For the establishment of cells stably expressing the circadian oscillation reporter, human iPSCs were transduced with mouse *Bmal1* promoter or *Per2* promoter-driven luciferase reporters (*Bmal1:luc*-pPB or *Per2:luc*-pPB). The human iPSCs were transfected using 16.5 µL of FuGENE 6 mixed with 1 µg of pCAG-PBase and 3 µg of *Bmal1:luc*-pPB or *Per2:luc*-pPB. The transfected cells were cultured for 14 days in a supplemented culture medium comprising 0.5 to 1 µg/mL puromycin.

In Vitro Differentiation

SNL feeder cells cultured with human iPSCs were eliminated by treatment with CTK solution: 0.25% (v/v) trypsin, 0.1 mg/mL collagenase IV, 1 mM $CaCl_2$, and 20% (v/v) Knockout SR (Invitrogen,

Carlsbad, CA). After the human iPSCs were trypsinized, embryoid bodies (EBs) were generated by harvesting 9000 or 12,000 cells and seeding them onto low-attachment 96-well plates (Lipidure Coat, NOF) in a differentiating medium, which comprised DMEM/F-12 (Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine (Nacalai Tesque), 110 μM StemSure 2-mercaptoethanol solution (Wako), and 0.5% penicillin-streptomycin (Nacalai Tesque) with 10 μM Y27632 (Wako). Six days later, EBs were plated onto gelatin-coated tissue culture 24-well plates and grown for several weeks. The media were exchanged every 4 to 5 days with the differentiating medium without Y27632.

Real-time Bioluminescence Analysis

Real-time bioluminescence analysis was performed according to the methods in previous reports (Umemura et al., 2013; Umemura et al., 2014). Briefly, the cells were seeded in 35-mm culture dishes or 24-well black plates, and the medium was replaced with Prime ESC medium or the differentiating culture medium without phenol red, comprising 0.2 mM luciferin (Promega, Madison, WI) and 15 mM HEPES. Cells were treated with 100 nM dexamethasone (Sigma, St. Louis, MO) or 10 μM forskolin (Wako) for synchronization. The dishes or plates were set on a turntable in an in-house fabricated real-time monitoring system developed by Dr. Takao Kondo (Nagoya University, Nagoya, Japan; Kiyohara et al., 2006). The bioluminescence from each dish or well was measured for 1 min at 20-min intervals.

Quantitative Real-time Polymerase Chain Reaction

Human iPSCs and the in vitro differentiated iPSCs were washed using ice-cold phosphate-buffered saline (PBS), and total RNAs were extracted using Isogen reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions and as described previously (Umemura et al., 2014). To remove the feeder cells from the iPSCs, the cells were treated with CTK solution, and then the mixed cell populations were seeded on gelatin-coated dishes and incubated for 20 min at 37 °C three times in Primate ESC medium. Nonattached iPSCs were used for the real-time polymerase chain reaction (RT-PCR) analysis. First-strand cDNAs were synthesized with 1 μg total RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative PCR analysis was performed using a StepOnePlus Real-time PCR system (Applied Biosystems, Foster City, CA) and iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). Standard PCR amplification protocols were applied followed by a dissociation-curve analysis to confirm specificity. Transcription levels in cultured cells were determined in triplicate and normalized to the level of 18S ribosomal RNA (rRNA). The following primer sequences were used: *BMAL1*, 5′-CATGCAACGCAATGTCCAG-3′ and 5′-GTGTATGGATTGGTGGCACCT-3′; *CLOCK*, 5′-CAACGCACACATAGGCCAT-3′ and 5′-TTGGGTTCTTCAACAGTGCA-3′;*PER1*,5′-CATCACGTCTGAGTACACACTTCAGA-3′ and 5′-AGGACGGCTGCCTGCTC-3′; *PER2*, 5′-TCCAGATACCTTTAGCCTGATGA-3′ and 5′-TTTGTGTGTGTCCACTTTCGA-3′; *CRY1*, 5′-AGAACAGATCCCAATGGAGACT-3′ and 5′-GTGCATTCCAGGGATCATAGA-3′; *CRY2*, 5′-CCTCCTCAGTCGGGATCAA-3′ and 5′-GCGGGAGTTCAGTTTCCTTA-3′; *NPAS2*, 5′-CTTCCCTGCCTCCCAACCATC-3′ and 5′-GGTCCCTGGCTGTTGTGAGTAG-3′;*OCT3/4*,5′-GACAGGGGGAGGGGAGGAGCTAGG-3′ and 5′-CTTCCCTCCAACCAGTTGCCCCAAA-3′; *SOX2*, 5′-AGCTACAGCATGATGCAGGA-3′ and 5′-GGTCATGGAGTTGTACTGCA-3′; *NANOG*, 5′-TGAACCTCAGCTACAAACAG-3′ and 5′-TGGTGGTAGGAAGAGTAAAG-3′; and 18S rRNA, 5′-CGCCGCTAGAGGTGAAATTC-3′ and 5′-CGAACCTCCGACTTTCGTTCT-3′.

Immunostaining

Immunofluorescence staining of human iPSCs and the differentiated cells was performed as described previously (Inada et al., 2014; Umemura et al., 2017). For the immunostaining of CLOCK protein, briefly, cells plated on coverslips were fixed in cold methanol. After washing with PBS, cells were blocked with 5% skim milk and then incubated using an anti-CLOCK antibody (CLSP4; Yoshitane et al., 2009) overnight at 4 °C. After washing with PBS, the cells were incubated with Alexa647-labeled anti-mouse IgG (1:1000; Jackson Laboratory, Bar Harbor, ME) as a secondary antibody. For NANOG, cells plated on coverslips were fixed with PBS comprising 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 anti-NANOG 1:200 (ReproCELL) as a primary antibody overnight at 4 °C. The cells were washed with PBS and were then incubated with Cy3-labeled anti-rabbit IgG (1:1000; Jackson Laboratory) as a secondary antibody. The nuclei were stained with Hoechst 33342 (Nacalai Tesque), and the cells were then washed with PBS and mounted with PermaFluor Mounting Medium (Thermo Scientific, Waltham, MA). The cells were observed under an LSM510 confocal laser-scanning

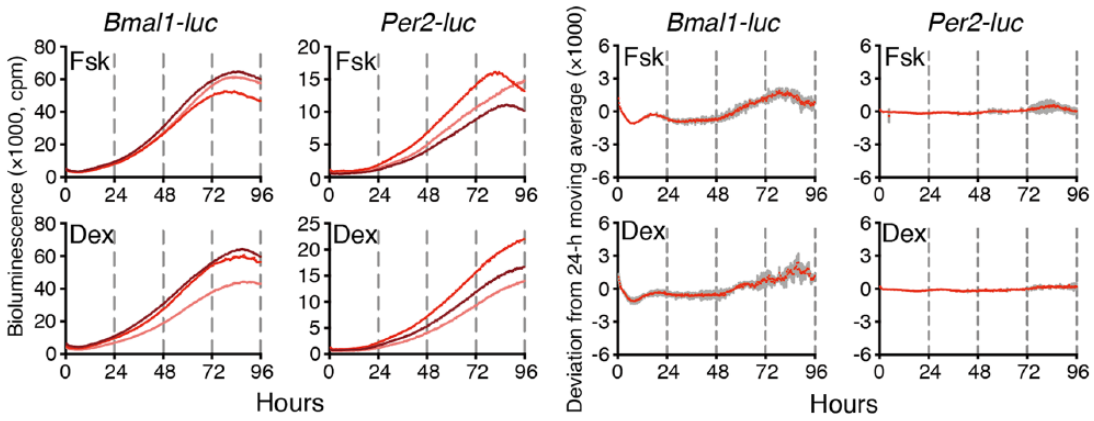

Figure 1. Human induced pluripotent stem cells (iPSCs) had no discernible circadian oscillation. Representative bioluminescent traces (left) and the averaged detrended traces (right, mean ± SD) in human iPSCs transduced with *Bmal1-luc* or *Per2-luc* reporters. Synchronization treatment was performed using forskolin (Fsk) or dexamethasone (Dex).

microscope (Zeiss, Oberkochen, Germany). The confocal microscopy setting for the CLOCK immunostaining is as follows: laser power 47.6%, master gain 600, digital offset 0, pinhole size 84 µm. For the quantification of CLOCK-immunostaining cells, the total cell number was counted using the Hoechst-staining image, and then the percentage of the CLOCK-immunostaining cells was calculated.

Data Analysis

For fast Fourier transform (FFT) analysis, the raw data were detrended by subtracting a 24-h moving average, and the relative spectral power density (relative power) at the peak within the range of 21 to 26 h was obtained by using Microsoft Excel (Redmond, WA).

RESULTS

Human iPSCs Had No Discernible Circadian Oscillation

First, we established human iPSCs transduced with *Bmal1* promoter-driven or *Per2* promoter-driven luciferase reporters. The human cell line iPSCs (*Bmal1-luc*) and iPSCs (*Per2-luc*) had no discernible circadian oscillation, despite synchronization treatments using dexamethasone or forskolin (Fig. 1). The result is consistent with our previous findings in mouse ESCs and iPSCs, in addition to mouse early embryos (Yagita et al., 2010; Umemura et al., 2013; Umemura et al., 2014; Umemura et al., 2017), and with a previous report using human ESCs (Dierickx et al., 2017).

In Vitro Differentiation of Human iPSCs

Subsequently, we differentiated the human iPSCs (*Bmal1-luc*) and iPSCs (*Per2-luc*) in vitro using EB formation, based on a method we developed previously using mouse ESCs (Umemura et al., 2013). After 6-day EB formation, EBs were plated onto gelatin-coated 24-well plates and cultured for several weeks (Fig. 2A). In vitro differentiation culture for 42 days resulted in considerable downregulation of pluripotent markers, including *NANOG, OCT3/4*, and *SOX2* (Fig. 2B). However, the bioluminescence from both *Bmal1-luc* and *Per2-luc* reporters in the 42-day differentiated cells showed only a slight circadian oscillation (Fig. 2C). Eventually, in vitro 90-day differentiation resulted in the emergence of apparent circadian oscillation from both *Bmal1-luc* and *Per2-luc* reporters, which had antiphase oscillations (Fig. 2C). FFT analysis of the circadian rhythmicity of the bioluminescence traces suggested gradual development of circadian oscillation during differentiation (Fig. 2D). Similar to that in mouse ESCs and iPSCs, the robust circadian oscillations were not observed immediately following the loss of pluripotent markers, and further in vitro differentiation culture induced the apparent circadian oscillations.

CLOCK Protein Was Posttranscriptionally Suppressed, and In Vitro Differentiation Culture Induced the CLOCK Protein Expression

To investigate the mechanisms that suppress the circadian oscillation in human iPSCs, CLOCK protein was examined because our previous study demonstrated that CLOCK protein expression was posttranscriptionally suppressed in mouse ESCs

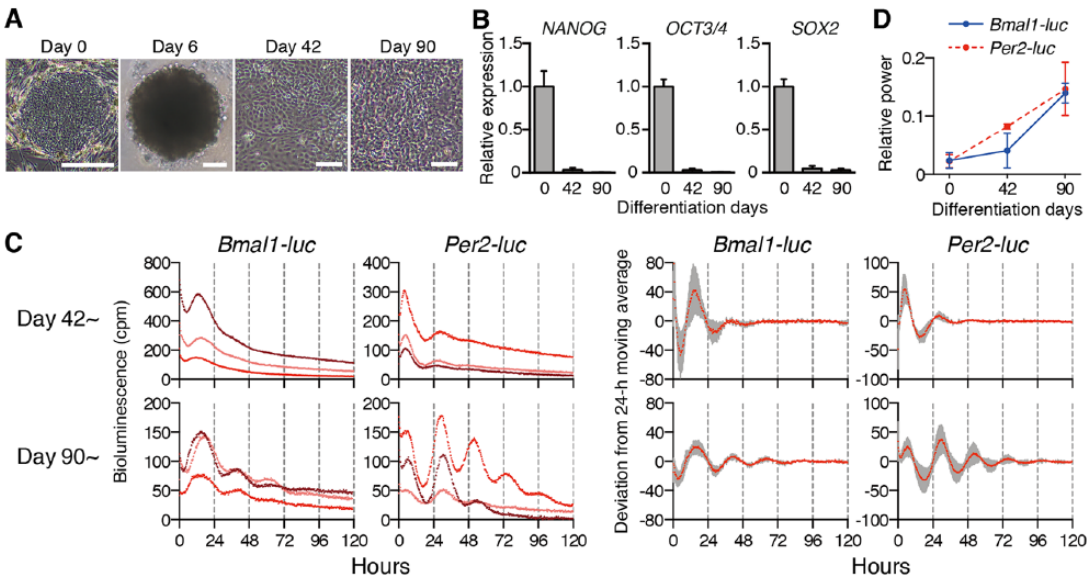

Figure 2. In vitro differentiated human induced pluripotent stem cells (iPSCs) exhibited robust circadian oscillation. (A) Morphological observation during in vitro differentiation of human iPSCs. Scale = 125 μm. (B) *NANOG*, *OCT3/4*, and *SOX2* gene expression during in vitro differentiation culture of human iPSCs. Each relative gene expression level of human iPSCs was set to 1. Data are presented with SD (n = 3 biological replicates). (C) Representative bioluminescent traces and the averaged detrended traces (mean ± SD) in 42- or 90-day differentiation cultures. In vitro 90-day differentiated human iPSCs exhibited circadian oscillation. Synchronization was performed using Fsk treatment. (D) Graphs of relative powers in circadian time of bioluminescence traces during in vitro differentiation (mean ± SD, n = 3 or 6).

and iPSCs as well as in mouse early embryos (Umemura et al., 2017). Immunostaining analysis revealed the suppression of CLOCK protein in undifferentiated human iPSCs, whereas the expression of NANOG, a pluripotent marker, was not suppressed (Fig. 3A,B). In addition, although the in vitro 42-day differentiated iPSCs exhibiting only slight circadian oscillations displayed no apparent signals from the immunostaining of CLOCK protein, in vitro 90-day differentiation induced CLOCK expression (Fig. 3B,C), in which the differentiated iPSCs exhibited apparent circadian oscillations (Fig. 2C). However, *CLOCK* mRNA was expressed at similar levels in both undifferentiated and in vitro differentiated human iPSCs, as well as in the other set of core clock genes except for *NPAS2* (Fig. 3D), indicating that CLOCK protein is posttranscriptionally suppressed in undifferentiated human iPSCs. These results are consistent with that in mouse ESCs, iPSCs, and early embryos (Umemura et al., 2017).

A paralogue of CLOCK, NPAS2, can compensate for CLOCK function (DeBruyne et al., 2007b, 2007a). The *NPAS2* expression level in undifferentiated human iPSCs and ESCs was extremely low compared with *CLOCK* expression level (Choi et al., 2015; Fig. 4), which is similar to that in mouse ESCs and early embryos (Umemura et al., 2017). Therefore, the posttranscriptional suppression of CLOCK protein expression is considered to be one of the reasons for the lack of circadian oscillator in undifferentiated human iPSCs.

DISCUSSION

In the present study, we investigated human circadian oscillation development using human iPSCs. We have previously reported that the posttranscriptional suppression of CLOCK protein expression in mouse ESCs, iPSCs, and early embryos is one of the critical mechanisms that inhibits the emergence of circadian oscillations in undifferentiated cells (Umemura et al., 2017). Consistent with the findings of this previous report, we observed that the expression of CLOCK protein in human iPSCs was also suppressed despite *CLOCK* mRNA expression (Fig. 3B,D). In vitro differentiation culture of human iPSCs induced the loss of the pluripotent markers (Fig. 2B). However, the robust circadian oscillation still did not develop in in vitro 42-day differentiation (Fig. 2C). In vitro differentiation culture for 90 days resulted in the emergence of apparent circadian oscillations and upregulation of CLOCK protein expression (Fig. 2C,D; Fig. 3B). *NPAS2* mRNA was also upregulated during in vitro differentiation

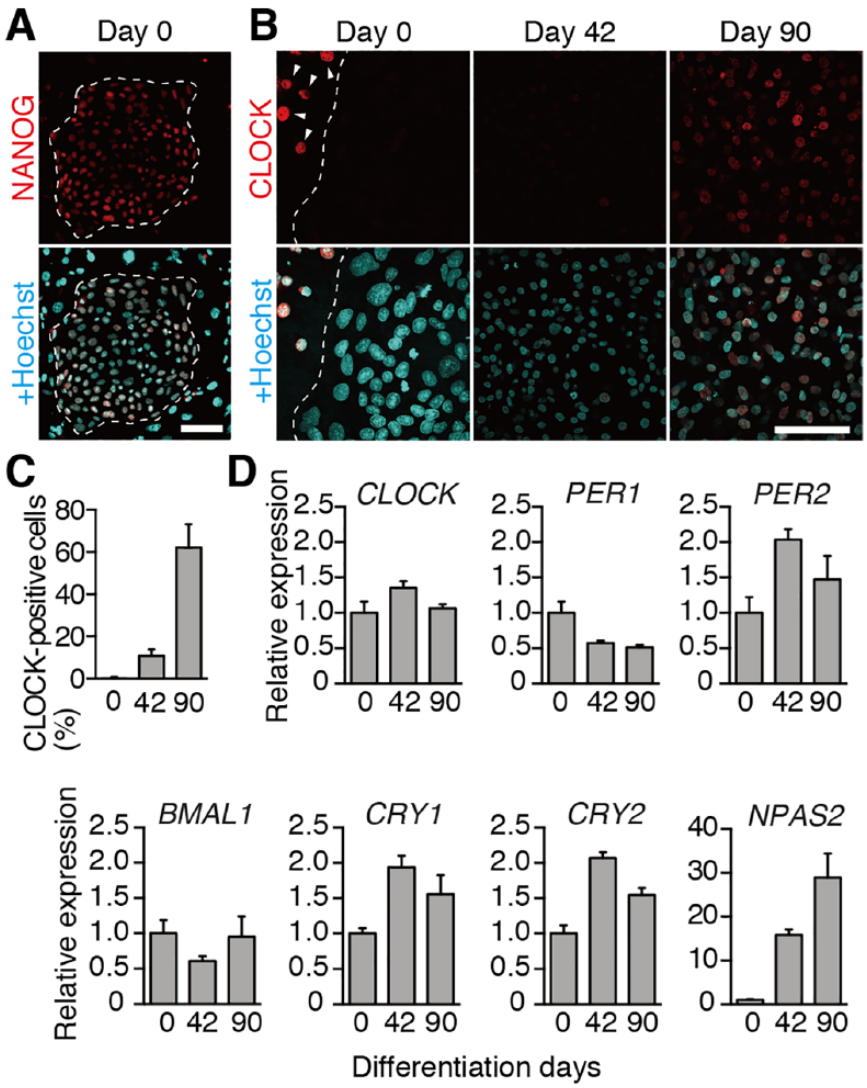

Figure 3. Posttranscriptional suppression of CLOCK protein in human induced pluripotent stem cells (iPSCs) and its appearance during in vitro differentiation culture of human iPSCs concomitant with the emergence of robust circadian oscillation. (A, B) Representative immunostaining of NANOG protein (A, $n = 3$ biological replicates) and CLOCK protein (B, $n = 5$-7 biological replicates) in human iPSCs (day 0) or in vitro differentiated human iPSCs (day 42 or 90). The human iPSC colony is surrounded by a dotted line. Arrowheads indicate feeder cells. The NANOG-negative differentiated feeder cells are morphologically quite different from human iPSCs and do not form colonies. These points were used for the discrimination between iPSCs and feeder cells. Scales = 100 µm. (C) The percentage of CLOCK-positive cells in the images acquired by the immunostaining analysis in Figure 3B. Mean ± SD. $n = 5$-7 biological replicates. (D) Quantitative real-time polymerase chain reaction of core clock genes during in vitro differentiation of human iPSCs. Data are shown with SD ($n = 3$ biological replicates). Each relative gene expression of undifferentiated human iPSCs was set to 1. The cells were not synchronized before the mRNA measurement.

(Fig. 3D), which may contribute to the emergence of apparent circadian oscillations during the differentiation. The robust circadian oscillations were not observed immediately following the loss of pluripotent markers, and further in vitro differentiation culture was required for the emergence of the apparent circadian oscillations. The manner of circadian oscillation emergence during the differentiation of human iPSCs is similar to that in in vitro differentiated mouse ESCs and iPSCs (Yagita et al., 2010; Umemura et al., 2013; Umemura et al., 2017).

Interestingly, although it is difficult to compare human pluripotent stem cells (PSCs) with mouse PSCs, human iPSCs required 3- to 4-fold longer differentiation culture periods for the emergence of robust circadian oscillation compared with that required by mouse PSCs, in which a ~14-day differentiation culture resulted in the emergence of the

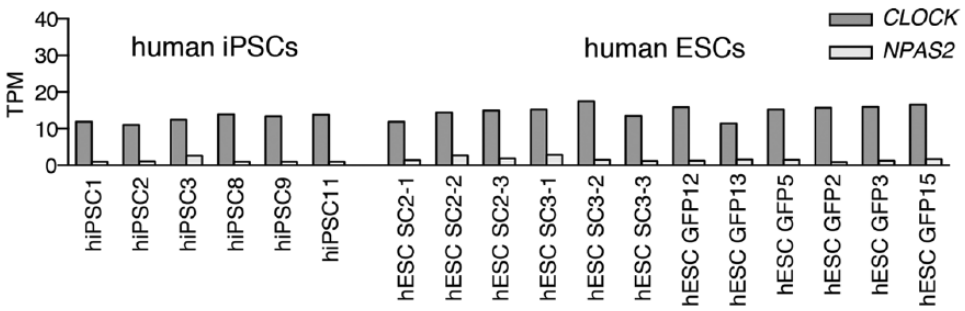

Figure 4. ***CLOCK*** **and** ***NPAS2*** **expression in undifferentiated human induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs). The expression levels (TPM) of** ***CLOCK*** **and** ***NPAS2*** **in each human iPSC or ESC were investigated by RNA sequencing in a previous report (GSE73211; Choi et al., 2015).**

robust circadian oscillation (Umemura et al., 2017). The gestation period in humans is much longer than that in mice. In addition, the doubling time for human iPSCs is 40 to 50 h (Takahashi et al., 2007), whereas that for both mouse iPSCs and ESCs is 17 to 19 h (Takahashi and Yamanaka, 2006). The differences in gestation period and doubling time could be associated with the difference in the time required for circadian oscillation to emerge between human and mouse cells, in which a longer period is required in human cells than in mouse cells.

Although ethical concerns preclude the probing of CLOCK protein suppression in early developmental stages in humans, we have recently reported similar findings of posttranscriptional suppression of CLOCK protein in some human epigenetic cancers, such as Wilms tumors and malignant rhabdoid tumors (Ohashi et al., 2018). It was suggested that the posttranscriptional suppression of CLOCK protein might exhibit not only the circadian clock impairment but also unknown pathophysiological significance, warranting further investigation.

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AUTHOR CONTRIBUTIONS

YU and KY designed the study; YU, IM, YT, NK, and KY conducted experiments and analyzed the data; YU and KY drafted the article.

CONFLICT OF INTEREST STATEMENT

The authors have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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