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Involvement of posttranscriptional regulation of *Clock* in the emergence of circadian clock oscillation during mouse development

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Circadian clock oscillation emerges in mouse embryo in the later developmental stages. Although circadian clock development is closely correlated with cellular differentiation, the mechanisms of its emergence during mammalian development are not well understood. Here, we demonstrate an essential role of the posttranscriptional regulation of *Clock* subsequent to the cellular differentiation for the emergence of circadian clock oscillation in mouse fetal hearts and mouse embryonic stem cells (ESCs). In mouse fetal hearts, no apparent oscillation of cell-autonomous molecular clock was detectable around E10, whereas oscillation was clearly visible in E18 hearts. Temporal RNA-sequencing analysis using mouse fetal hearts reveals many fewer rhythmic genes in E10–12 hearts (63, no core circadian genes) than in E17–19 hearts (483 genes), suggesting the lack of functional circadian transcriptional/translational feedback loops (TTFLs) of core circadian genes in E10 mouse fetal hearts. In both ESCs and E10 embryos, CLOCK protein was absent despite the expression of *Clock* mRNA, which we showed was due to *Dicer/Dgcr8*-dependent translational suppression of CLOCK. The CLOCK protein is required for the discernible molecular oscillation in differentiated cells, and the posttranscriptional regulation of *Clock* plays a role in setting the timing for the emergence of the circadian clock oscillation during mammalian development.

circadian clock | ontogeny | cellular differentiation | CLOCK | posttranscriptional regulation

In mammals, the circadian clock controls temporal changes of physiological functions such as sleep/wake cycles, body temperature, and energy metabolism throughout life (1–3). Although the suprachiasmatic nucleus (SCN) functions as a center of circadian rhythms, most tissues and cells and cultured fibroblast cell lines contain an intrinsic circadian oscillator controlling cellular physiology in a temporal manner (4–7). The molecular oscillator comprises transcriptional/translational feedback loops (TTFLs) of circadian genes. Two essential transcription factors, CLOCK and BMAL1, heterodimerize and transactivate core circadian genes such as *Period* (*Per1*, *2*, *3*), *Cryptochrome* (*Cry1*, *2*), and *Rev-Erbα* via E-box enhancer elements. PER and CRY proteins in turn repress CLOCK/BMAL1 activity and express these circadian genes cyclically (8, 9). REV-ERBα negatively regulates *Bmal1* transcription via the RORE enhancer element, driving antiphasic expression patterns of *Bmal1* (10, 11).

Although circadian clocks reside throughout the body after birth, mammalian zygotes, early embryos, and germline cells do not display circadian molecular rhythms (12–14), and the emergence of circadian rhythms occurs gradually during development (15–17). In addition, it has been elucidated that embryonic stem cells (ESCs) and early embryos do not display discernible circadian molecular oscillations, whereas circadian molecular oscillation is clearly observed in in vitro-differentiated ESCs (18, 19). Moreover, we have shown that circadian oscillations are abolished when differentiated cells are reprogrammed to regain pluripotency through reprogramming factor expression (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*) (19), indicating that circadian clock development in mammalian cells is closely correlated with the cellular differentiation process.

Supporting these findings, perturbation of the cellular differentiation process of ESCs via DNA methyltransferase (DNMT, i.e., *Dnmt1*, *3a*, *3b*) deficiency during differentiation results in the abolishment of circadian clock development (20). As the misregulation of DNMTs affects global gene expression and induces dysdifferentiation through the impairment of epigenetic and transcriptional programs in various cell types (21–24), the failure of circadian clock development in these models indicates that adequately regulated cellular differentiation is indispensable for circadian clock development in mammalian cells (19, 20). Also, we recently found that *Karyopherinα2* (*Kpna2*) overexpression disrupts cellular differentiation-coupled circadian clock development (20). KPNA2 was originally identified as an importin α subunit that is highly expressed in ESCs (25), and it plays a distinct role in maintaining pluripotency in ESCs by promoting the nuclear entry of OCT3/4 and preventing the nuclear entry of OCT6, which leads to differentiation (25, 26). Therefore, these findings provide further support that circadian clock development requires adequate cellular differentiation.

Significance

Circadian clocks reside in each cell level throughout the body in mammals. Intrinsic cellular circadian clocks develop cell autonomously during the cellular differentiation process. However, mechanisms controlling the emergence of cellular circadian clock oscillation in vivo are not fully understood. Here, we show that Dicer/Dgcr8-mediated posttranscriptional mechanisms control the CLOCK protein expression in both mouse fetal hearts and in vitro differentiating ES cells, which contributes to the emergence of circadian clock in mammalian cells. This event occurs after cell lineage determination into hearts or loss of pluripotent stem cell markers in differentiating ES cells, suggesting the cellular differentiation-coupled clock development may be conducted by a two-step program consisting of cellular differentiation and subsequent establishment of circadian transcriptional/translational feedback loops.

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Cellular differentiation is a process to establish the cell lineage-specific gene-expression network regulated by global epigenetic and transcriptional programs (27, 28). Therefore, it is reasonable to predict that the emergence of circadian clock oscillation should be observed along with the cell lineage determination. However, previous studies showed that the intrinsic molecular oscillation appeared only around E13~18 in mice fetal tissues such as heart and liver (29–32). Moreover, we previously demonstrated that the emergence of circadian clock oscillation during the in vitro differentiation of ESCs required ≥14 d in culture after the differentiation started (19, 20). Although pluripotent markers disappear by day 7 of differentiation in culture, circadian molecular oscillation had not yet emerged in these cells (19, 20). These findings suggest that additional mechanisms after the establishment of the cell lineage-specific gene-expression network are likely required for the activation of the mammalian circadian clock.

In this study, we investigated the mechanism that starts the circadian molecular oscillator cycle during the developmental process using mouse embryonic hearts and ESCs as models. Circadian gene reporter studies and temporal RNA-sequencing (RNA-seq) analysis revealed a lack of core circadian TTFLs in E10–12 hearts as well as in the early differentiation stage (day 7) of ESCs. Next, using ESCs as a model system of differentiation-coupled circadian clock development, we showed that the gradual appearance of CLOCK protein during ESC differentiation correlated with the emergence of molecular oscillation. Mechanistically, we showed that the *Dicer*/*Dgcr8*-mediated posttranscriptional suppression of CLOCK protein contributes to the late development of the circadian clock oscillation. These findings indicate that the posttranscriptional regulation of *Clock* may play an important role for the emergence of circadian clock oscillation during mouse development.

Results

Cell-Autonomous Circadian Clock Has Not Developed in E9.5–10 Fetal Hearts. We first investigated circadian clock oscillation during mouse development after organogenesis. Hearts obtained at

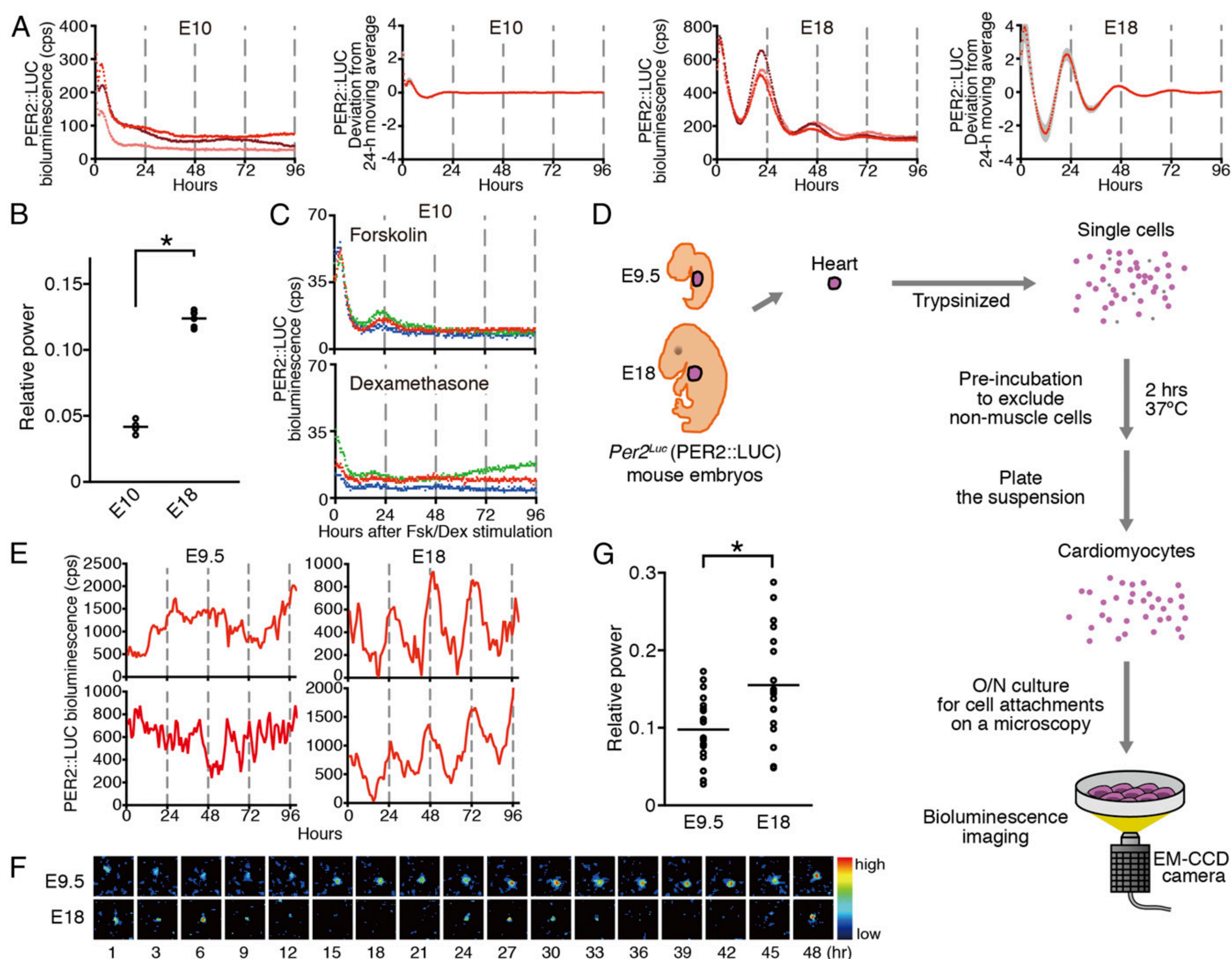

Fig. 1. Circadian PER2::LUC oscillation has not yet developed in E10 mouse hearts. (*A*) Bioluminescence traces from ex vivo culture of the embryonic hearts. Representative raw data (*Left*) and averaged detrended data by subtracting a 24-h moving average (*Right*) are shown for E10 and E18 hearts. Data are shown as mean ± SEM, $n = 4$ or 6 biological replicates. The *x* axes indicate the time after culture in the supplemented DMEM/Ham's F-12 medium containing luciferin without Dex/Fsk stimulation. (*B*) FFT spectral power analysis of the bioluminescence from the mouse embryonic heart culture. The bars denote the mean ($n = 4$ or 6 biological replicates, two-tailed *t* test, $^*P < 0.01$). (*C*) Bioluminescence traces from the E10 hearts stimulated by Fsk and Dex. The *x* axes indicate the time after stimulation. Data from three biological replicates are represented in different colors. (*D*) Scheme of the dispersed cell cultures of E9.5 and E18 $Per2^{Luc}$ embryos for single-cell bioluminescence imaging. (*E* and *F*) Representative single-cell bioluminescence traces (*E*) and image sequences (*F*) from the dispersed cardiomyocytes cultured without Dex/Fsk stimulation. The *x* axes indicate the time after recording. (*G*) FFT spectral power analysis of single-cell bioluminescence ($n = 19$ or 20 biological replicates, two-tailed *t* test, $^*P < 0.01$).

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E10 did not display discernible circadian molecular oscillations, whereas E18 hearts exhibited apparent daily bioluminescence rhythms (Fig. 1 *A* and *B*). Synchronization stimulation using forskolin (Fsk) and dexamethasone (Dex) failed to induce detectable bioluminescence oscillation (Fig. 1*C*). A single-cell-level analysis using cardiomyocytes prepared from E9.5 and E18 fetal hearts indicated that cardiomyocytes derived from E9.5 embryos did not express apparent circadian $Per2^{Luc}$ bioluminescence rhythms, whereas circadian oscillation was observed in E18 cardiomyocytes (Fig. 1 *D*–*G*). These results clearly reveal that the heart tissues of ~E10 mouse fetuses do not have a functional cell-autonomous circadian clock.

Circadian Rhythm of Global Gene Expression Is Not Yet Developed in E10–12 Mouse Fetal Hearts in Vivo. Although the cell-autonomous circadian clock did not cycle in E10 heart tissues, it might be possible that maternal circadian rhythms entrain or drive the fetal circadian clock in vivo. Therefore, we performed temporal RNA-seq analysis to investigate the circadian rhythmicity of global gene expression in E10–12 and E17–19 fetal hearts. Pregnant mice were housed under a 12-h:12-h light-dark (LD12:12) cycle (6:00 AM light onset) and then were subjected to constant darkness for 36 h before sampling. Sampling of fetal hearts was performed every 4 h for 44 h (two cycles) from circadian time 0 (CT0, i.e., 6:00 AM) at the E10 or E17 stage (Fig. 2*A*). We used E10–12 and

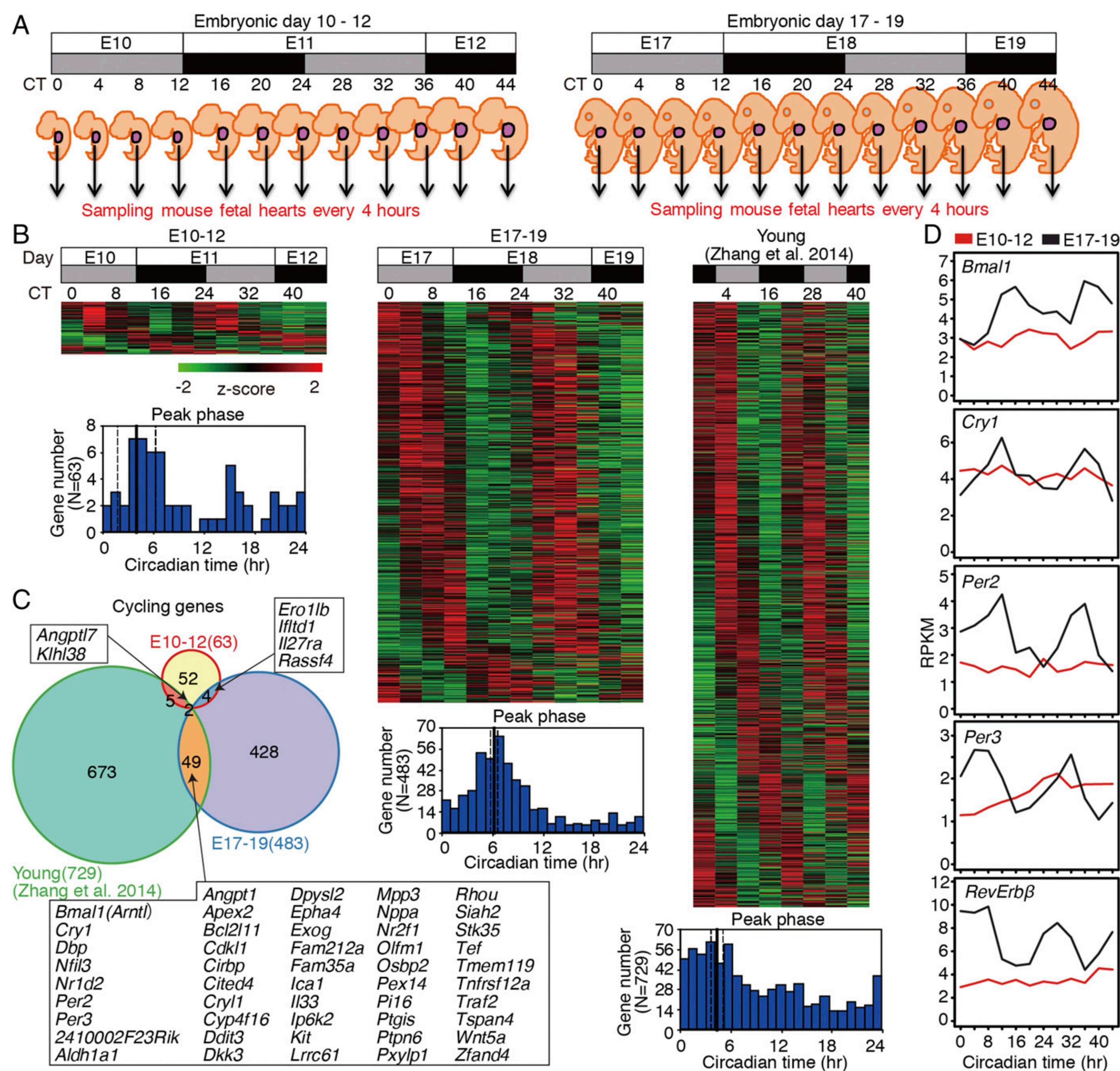

Fig. 2. RNA-seq analysis of circadian gene expression in the mouse hearts. (*A*) Schematic representation of mouse heart sampling. The gray and black boxes indicate the subjective day and night, respectively, and the circadian time and embryonic day are indicated. (*B*) Heatmap view of cycling genes. Each gene is represented as a horizontal line ordered vertically by phase as determined by MetaCycle. The phase of each transcript rhythm is represented in the histogram plot. (*C*) Venn diagram of cycling genes in the mouse hearts. (*D*) Cyclic expression of circadian genes. RNA expression levels at E10–12 and E17–19 are indicated by red and black traces, respectively. The expression of *Bmal1* (*Arntl*), *Cry1*, *Per2*, *Per3*, and *Rev-Erbβ* (*Nr1d2*) is circadian in E17–19 hearts (MetaCycle; $P < 0.05$).

E17–19 mouse hearts to perform polyA-selected RNA sequencing (mRNA-seq) (Dataset S1). Cardiomyocyte markers such as *Mef2c*, *Nkx2.5*, and *Tbx5* were expressed in both E10–12 and E17–19 mouse fetal hearts, confirming the lineage commitment of the RNA-seq samples we used (Fig. S1). In young adult mice, ≈6% of genes in the hearts display circadian expression (33). Similarly, 4.0% (483 genes) of expressed genes in E17–19 hearts exhibited circadian expression rhythms (Fig. 2*B*). On the other hand, only 63 genes (0.5%) were rhythmically expressed in E10–12 hearts (Fig. 2*B* and Dataset S2). Only six cycling genes in E10–12 and E17–19 overlapped (Fig. 2*C*), and none of these was a known circadian-controlled gene. Conversely, several essential circadian genes and canonical clock-output genes such as *Bmal1*, *Cry1*, *Per2*, *Per3*, *Nr1d2* (*Rev-erbβ*), and *Dbp* were detected as rhythmic in the hearts of E17–19 fetuses and young adult mice (Fig. 2 *C* and *D* and Datasets S2 and S3).

As the temporal profiles of circadian genes observed in the RNA-seq analysis were validated by qPCR analysis (Fig. S2*A*), evidence of circadian oscillation in core circadian genes was not detected in E10–12 hearts in vivo. Comparing the gene-expression levels between E10–12 and E17–19 fetal hearts, ≈2,700 genes exhibited altered expression: 1,309 were down-regulated, and 1,409 were up-regulated in E17–19 hearts (Fig. S2 *B* and *C*); however, the expression levels of core circadian genes (excluding *Npas2*) were not changed dramatically (Fig. S2*D*). These results indicate that core circadian TTFLs are not yet developed in E10–12 mouse fetal hearts in vivo despite the expression of essential circadian clock genes. This may also indicate that cell lineage determination such as cardiomyocyte differentiation is insufficient for the emergence of core circadian gene oscillations and that subsequent mechanism(s) are required for the completion of mammalian circadian clock development.

Emergence of Circadian Clock Oscillation Along with Posttranscriptionally Regulated CLOCK Expression During Differentiation Culture of ESCs. Next, we examined whether in vitro differentiation culture of pluripotent stem cells (PSCs) could recapitulate and be a useful model system of late mammalian circadian clock development. To this end, not only ESCs but also other types of PSC lines such as induced PSCs (iPSCs) (34) and multipotent germline stem cells (mGSCs) (35) were tested for their ability to undergo differentiation-coupled circadian clock development in vitro. The PSC lines did not display circadian oscillation of *Bmal1* promoter-driven luciferase (*Bmal1-luc*) bioluminescence (Fig. 3*A*). Moreover, although in vitro differentiation culture for 7 d resulted in the loss of pluripotent markers (*Nanog*, *Oct3/4*, and *Sox2*), none of the cell lines exhibited circadian clock oscillation at this differentiation state (Fig. 3 *A* and *B*). Conversely, molecular oscillation started to emerge after 14 d of differentiation, and all PSCs induced robust circadian clock oscillation after 28 d of differentiation culture (Fig. 3 *A* and *C*). These findings strongly suggest that a common principle controls differentiation-coupled circadian clock development in mammalian cells and that in vitro differentiation of PSCs recapitulates the late emergence of molecular clock oscillation. Therefore, we used ESCs as a model system to investigate the mechanism(s) regulating circadian clock development in mammals.

By surveying the expression of core clock proteins in PSCs, we found that CLOCK protein was absent in all PSCs (Fig. 4 *A*–*C*). Immunofluorescence analysis during in vitro differentiation culture revealed that the CLOCK was gradually detected beginning 14 d after differentiation, which correlates well with the timing of the emergence of molecular oscillation (Fig. 4 *D* and *E*). Interestingly, *Clock* mRNA was constitutively expressed throughout differentiation culture and in undifferentiated ESCs (Fig. 4*F* and Fig. S2*E*) (19, 20), indicating that posttranscriptional regulation controlled differentiation-coupled CLOCK protein expression.

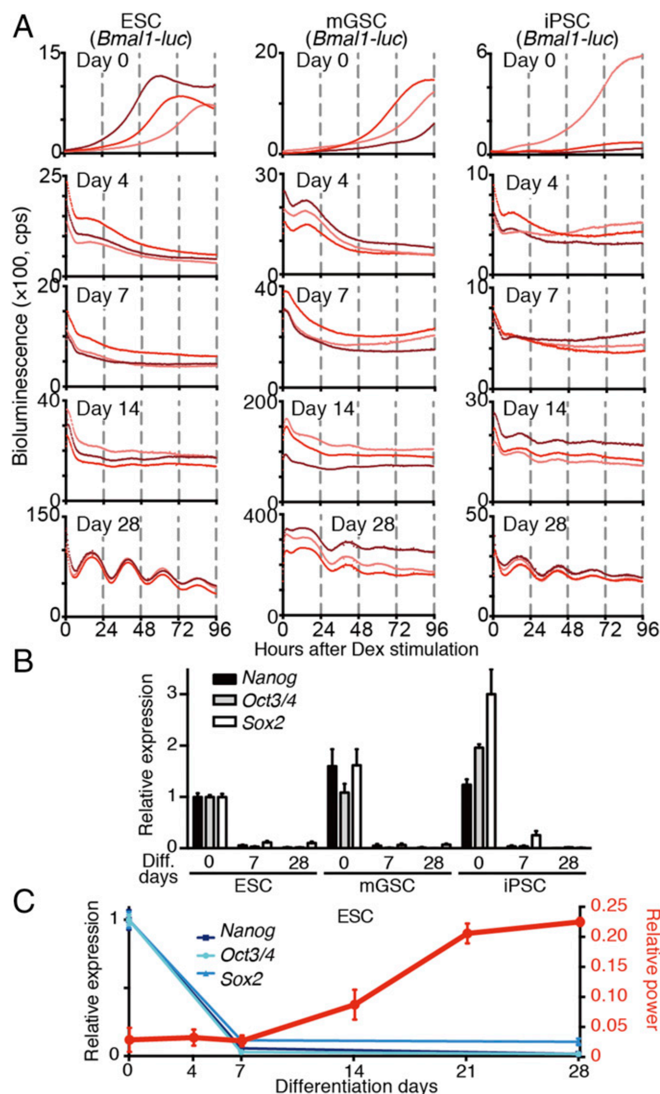

Fig. 3. Differentiation-coupled circadian clock development PSCs emerged slowly after the complete loss of pluripotent markers. (*A*) Representative raw bioluminescence traces. All PSCs carrying *Bmal1-luc* reporters were differentiated in vitro for the indicated days. No PSCs exhibited any apparent circadian oscillation before day 14 of differentiation culture. Weak oscillation was detected at day 14, and apparent oscillation was observed at day 28 in all PSCs. (*B*) qPCR analysis of the pluripotent markers *Nanog*, *Oct3/4* (also known as *Pou5f1*), and *Sox2* in undifferentiated PSCs and 7- and 28-d in vitro-differentiated PSCs. Data are presented as the mean ± SD (n = 3 biological replicates). (*C*) Graphs of the relative expression levels of pluripotent markers of ESCs indicated in *B* (blue lines, mean ± SD, n = 3 biological replicates) and relative powers of circadian time of bioluminescence traces in ESCs during in vitro differentiation (red lines, mean ± SD, n = 4–6 biological replicates).

Next, to elucidate the importance of CLOCK expression for the emergence of circadian oscillation, we generated *Clock*- and/or *Npas2*-deficient $Per2^{Luc}$ ESC lines (Fig. S3 *A*–*D*). In an in vitro differentiation assay, *Clock* played a dominant role in the emergence of circadian clock oscillation (Fig. S3 *B* and *C*), which was compatible with previous studies demonstrating the importance of *Clock* for circadian rhythms in most peripheral tissues (36). *Npas2* can compensate for *Clock* function in neuronal tissues such as the SCN (32, 36, 37). *Npas2* was almost undetectable in undifferentiated ESCs, and although a low level of *Npas2* expression was detectable in the 28-d differentiated cells (Fig. S3*E*),

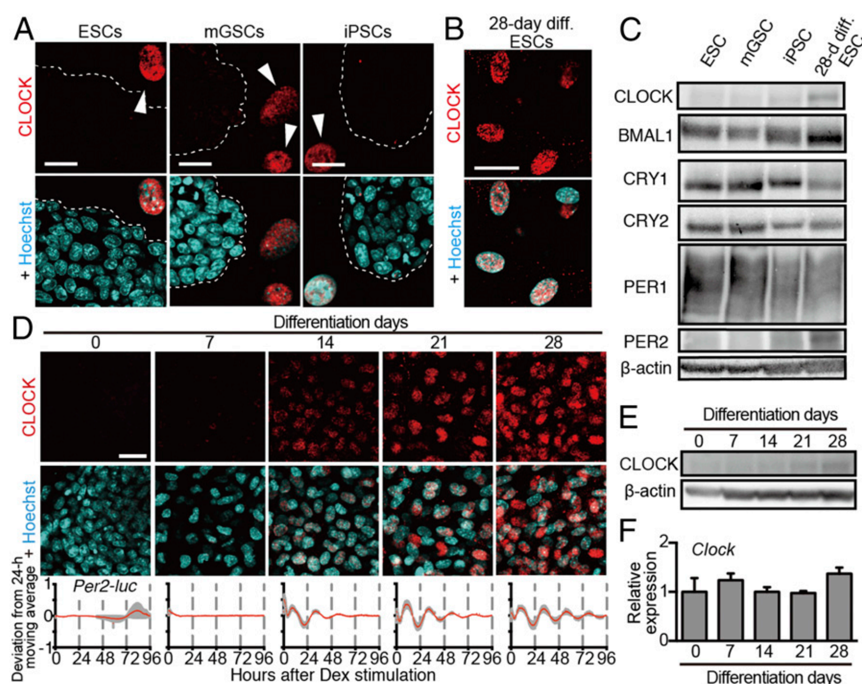

Fig. 4. Absence of CLOCK in PSCs and its gradual appearance during the in vitro differentiation of ESCs concomitant with the emergence of circadian oscillation. (*A* and *B*) Representative immunostaining of CLOCK protein in ESCs, mGSCs, iPSCs, (*A*) and 28-d differentiated (28-d diff) ESCs (*B*). Immunostaining (red) and Hoechst nuclear staining (blue) are shown. Feeder MEFs are indicated by arrowheads in *A*. PSCs are surrounded by dotted lines in *A*. (*C*) Western blots of core circadian proteins in ESC, mGSC, iPSC, and 28-d diff ESCs (n = 1 or 2 biological replicates). (*D*) Temporal expression of CLOCK protein during differentiation. (*Top* and *Middle*) Representative immunostaining (n = 2–4 biological replicates) is shown as described in *A*. (*Bottom*) Averaged bioluminescence traces (SEM, n = 3 or 6 biological replicates) were detrended by subtracting the a 24-h moving average of in vitro-differentiated ESCs carrying *mPer2* promoter-driven luciferase reporters at the indicated times. (*E* and *F*) Representative Western blot analysis of CLOCK protein (n = 2 biological replicates) (*E*) and qPCR analysis of *Clock* mRNA (*F*) for in vitro-differentiated ESCs for the indicated days. Data are shown with the SD (n = 3 biological replicates).

the disruption of *Npas2* showed only a subtle effect on the circadian oscillation in the differentiated cells (Fig. S3 *B–E*). Moreover, doxycycline-dependent *Clock* expression rescued circadian clock oscillation in *Clock*/*Npas2* doubly deficient (dKO) ESCs after differentiation culture (Fig. S4). These results support the importance of CLOCK protein expression for circadian clock development during ESCs differentiation.

Acceleration of Circadian Clock Development by CLOCK Overexpression During in Vitro Differentiation. Next, we determined whether CLOCK protein expression can evoke the emergence of circadian clock oscillation during in vitro differentiation. Doxycycline-inducible $Per2^{Luc}$ ESCs overexpressing *Clock* (*Clock* OX) were used for in vitro differentiation assays. Expression of pluripotent markers (*Nanog*, *Oct3/4*, and *Sox2*) rapidly decreased to almost undetectable levels at day 6 of in vitro differentiation culture in both $Per2^{Luc}$ and *Clock* OX ESCs (Fig. 5*A*), suggesting that *Clock* overexpression did not influence the cellular differentiation process. We then compared the development speed of circadian molecular oscillators during in vitro differentiation between the cells with or without *Clock* overexpression. Since *Clock* OX ESCs showed leaking *Clock* expression without doxycycline, WT $Per2^{Luc}$ ESCs were used as a control. Although a Western blot confirmed that the CLOCK protein was expressed throughout the differentiation culture in *Clock* OX cells (Fig. 5*B*), CLOCK overexpression failed to evoke $Per2^{Luc}$-driven circadian bioluminescence in undifferentiated ESCs (Fig. 5*C*). An in vitro differentiation assay revealed that the $Per2^{Luc}$-driven circadian bioluminescence rhythm had appeared earlier in *Clock* OX cells than in $Per2^{Luc}$ cells (Fig. 5*C*), and the Fast Fourier Transform (FFT) relative power of *Clock* OX cells at day 8 and day 9 during differentiation was significantly higher than that of $Per2^{Luc}$ cells (Fig. 5*D*). These results indicate that the early expression of CLOCK protein by *Clock* overexpression during the differentiation of ESCs accelerates the circadian clock development. Moreover, the lack of a significant increase in the FFT relative power in ESCs and in *Clock* OX cells at day 4 suggested that the CLOCK expression was not solely sufficient for the induction of circadian clock cycling.

CLOCK Expression in E10 Mouse Fetal Hearts Is also Posttranscriptionally Suppressed. Using ESCs, we identified the contribution of posttranscriptional inhibition of CLOCK expression to the emergence of circadian clock oscillation during the differentiation culture. To examine whether this mechanism is at play during mammalian circadian clock development in vivo, we investigated CLOCK expression in mouse embryos and fetuses. Immunohistochemistry revealed that E6.5 embryos and E10 fetal hearts did not express CLOCK, whereas apparent signals against CLOCK were observed in the nuclei of E17.5 fetal hearts and maternal uterus tissue (decidua) surrounding E6.5 embryos (Fig. 6*A*). Western blotting also confirmed that CLOCK expression was hardly detectable in E10.5 fetal heart, whereas clear expression of CLOCK was observed in mouse embryonic fibroblasts (MEFs) as well as E17.5 fetal heart (Fig. 6*B*). RNA-seq data using E10–12 and E17–19 fetal hearts revealed that *Clock* mRNA was constitutively expressed at both developmental stages (Fig. 6*C*), indicating that the suppression of CLOCK in E10 fetal hearts was most likely due to posttranscriptional regulation, as observed in ESCs. Because *Clock* and *Npas2* are indispensable to generate circadian rhythm (36, 37), and *Npas2* mRNA was not expressed in E10–12 fetal hearts (Fig. 6*D*), the lack of CLOCK expression by the posttranscriptional inhibition is at least one of the reasons for the absence of cell-autonomous circadian clock oscillation in E10 hearts.

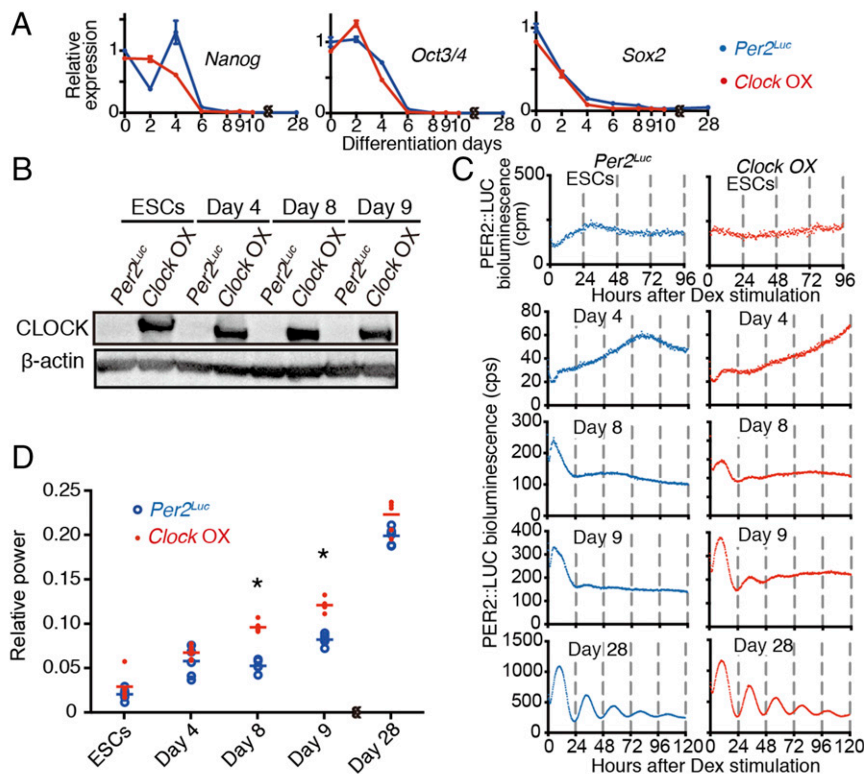

Fig. 5. CLOCK expression was insufficient for the circadian clock cycling in the undifferentiated ESCs. (*A*) Temporal expression profiles of *Nanog*, *Oct3/4*, and *Sox2* genes during in vitro differentiation culture of *Per2*^{Luc} (blue traces) and *Clock*-overexpressed *Per2*^{Luc} (*Clock* OX, red traces) ESCs. Data are shown with SEM ($n = 3$ biological replicates). (*B*) Expression profile of CLOCK protein in ESCs and at day 4, 8, and 9 after in vitro differentiation of *Per2*^{Luc} and *Clock* OX ESCs ($n = 1$). (*C* and *D*) Representative bioluminescence traces (*C*) and FFT spectral power analysis (*D*) of ESCs and 4-, 8-, 9-, and 28-d differentiated ESCs. Bars indicate the mean ($n = 6$ biological replicates, two-way ANOVA followed by Bonferroni's post hoc comparisons tests, $^*P < 0.01$).

Dicer/Dgcr8-Mediated Posttranscriptional Mechanism Suppresses Clock Translation. As E10 mouse fetal hearts and premature differentiated ESCs, both lacking detectable cell-autonomous circadian oscillation, did not express CLOCK, we further investigated the mechanism of the posttranscriptional regulation of *Clock*. Using the open database for genome-wide translational efficiency in ESCs based on ribosome profiling reported by Ingolia et al. (38), we found that the translational efficiency of *Clock* mRNA in undifferentiated ESCs is extremely low compared with that of other circadian genes (Fig. S5). This supports the hypothesis that the posttranscriptional regulation of *Clock* inhibits its translation in ESCs.

Because DICER- and DGCR8-mediated biosynthesis of miRNAs plays essential roles in the inhibition of the translation of various genes (39, 40), we next examined the effects of genetic ablation of *Dicer* and *Dgcr8* on CLOCK expression in ESCs. Both immunofluorescence and Western blot analysis confirmed the presence of CLOCK proteins in *Dicer*$^{-/-}$ and *Dgcr8*$^{-/-}$ ESCs (Fig. 7 *A* and *B*). These results revealed that the DICER/DGCR8-dependent posttranscriptional mechanism regulated CLOCK expression in ESCs. To measure circadian clock oscillation in the *Dicer*$^{-/-}$ and *Dgcr8*$^{-/-}$ ESCs, mouse *Per2* promoter-driven luciferase reporters were introduced. We observed that the circadian clock did not oscillate in *Dicer*$^{-/-}$ and *Dgcr8*$^{-/-}$ ESCs despite CLOCK expression in these cells (Fig. 7*C*). Since the loss of *Dicer* or *Dgcr8* in ESCs causes differentiation defects in vivo and in vitro (41, 42), and ESC markers such as *Nanog*, *Oct3/4*, and *Sox2* were still expressed after 28-d in vitro differentiation of *Dicer*$^{-/-}$ and *Dgcr8*$^{-/-}$ cells (Fig. S6*A*), the lack of circadian oscillation in these cells is due to the failure of differentiation (Fig. S6 *B*–*D*). These results reveal that the adequate cellular differentiation process is a prerequisite for the emergence of circadian oscillation before CLOCK expression.

To validate that the *Clock* UTR contains *cis*-regulatory elements of posttranscriptional suppression, we compared expression levels of *Clock* 5′ or 3′ UTR-fused *luciferase* in undifferentiated ESCs with 28-d in vitro-differentiated ESCs. Both *Clock* 5′ and 3′ UTRs significantly reduced luciferase activities in ESCs compared with 28-d in vitro-differentiated ESCs (Fig. S7*A*). The luciferase activities of *Clock* 3′ UTR-fused *luciferase* reporter were significantly increased in both *Dicer*$^{-/-}$ and *Dgcr8*$^{-/-}$ ESCs compared with WT ESCs, although the *Clock* 5′ UTR-fused *luciferase* reporter showed a subtle effect in both *Dicer*$^{-/-}$ and *Dgcr8*$^{-/-}$ ESCs (Fig. S7*B*). These results suggest that the *Clock* 3′ UTR possesses the *cis*-elements for Dicer/Dgcr8-mediated posttranscriptional inhibition of CLOCK expression and works more efficiently than the *Clock* 5′ UTR.

Next, we extracted candidate miRNAs among the predicted miRNAs targeting the UTRs of *Clock* mRNA (43). To extract the candidate miRNAs, we used recently obtained RNA-seq data from WT and *Dnmt* (*Dnmt1*, *Dnmt3a*, *Dnmt3b*)-deficient ESC lines (20). In *Dnmt1*$^{-/-}$ and *Dnmt1*$^{-/-}$ *Dnmt3a*$^{-/-}$ *Dnmt3b*$^{-/-}$ triple-knockout (TKO) ESCs, neither CLOCK expression nor circadian clock oscillation was detected after differentiation culture for 28 d (Fig. 8*A*). We previously showed that ESC markers (*Nanog*, *Oct3/4*, and *Sox2*) were still expressed in the *Dnmt1*$^{-/-}$ and TKO cells after 28-d differentiation culture (20), suggesting that the lack of CLOCK and circadian rhythm in these cells may also be due to the failure of adequate differentiation. As *Clock* mRNA is constitutively expressed in these *Dnmt*-deficient cells throughout differentiation culture (Fig. S7*C*) (20), CLOCK expression is expected to be also inhibited via a posttranscriptional mechanism in these cells as observed in the undifferentiated WT ESCs and E10 mouse hearts. Using the RNA-seq data, we extracted eight candidate miRNAs and two miRNA

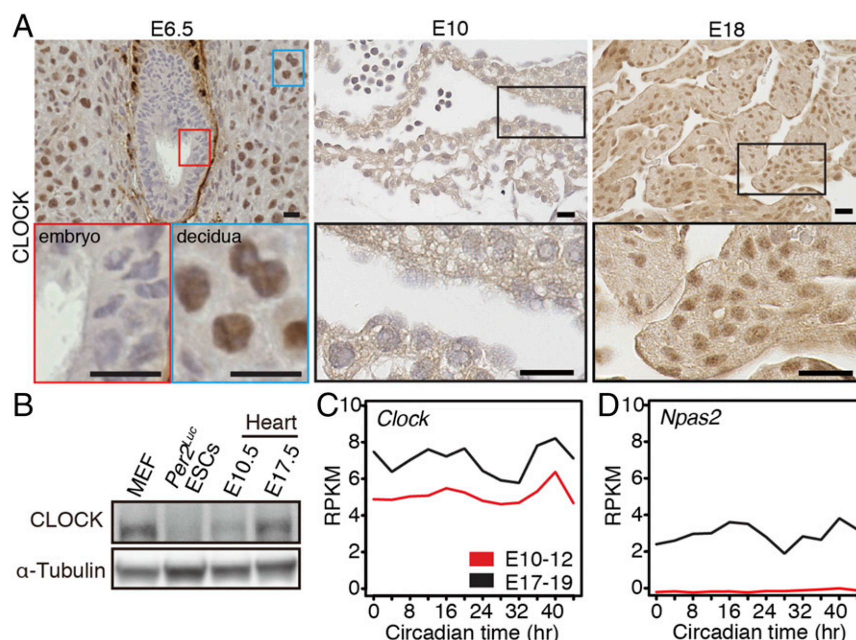

Fig. 6. The absence of CLOCK expression in early embryos. (*A*) Representative immunohistochemistry of CLOCK in embryos, decidua, and embryonic hearts on the indicated days ($n = 3$ biological replicates). (Scale bars, 20 μm.) (*B*) Representative Western blot analysis of CLOCK proteins in MEFs, $Per2^{Luc}$ ESCs, and E10.5 and E17.5 hearts ($n = 2$ biological replicates). (*C* and *D*) mRNA expression levels of *Clock* (*C*) and *Npas2* (*D*) in embryonic hearts using RNA-seq data.

clusters that were commonly up-regulated in ESCs and the *Dnmt*-deficient cells (designated "nonrhythmic cells") lacking CLOCK expression and circadian clock oscillation (Fig. 8*B*).

Luciferase reporter-based posttranscriptional repression assays using the *Clock* 5′ or 3′ UTR-fused *luciferase* mRNA expression constructs revealed that *Mir1306*, *Mir290-295*, and *Mir17hg* statistically significantly inhibited the translational efficiency (Fig. 8*C*). In mouse embryos, 45 miRNAs were up-regulated in E10–12 hearts relative to their expression in E17–19 hearts (Fig. 8*D*). Among them, 10 miRNAs were also up-regulated in nonrhythmic cells including ESCs (Fig. 8*E*). Strikingly, two functionally identified miRNA genes, *Mir1306* and *Mir17hg*, were highly and constitutively expressed in E10–12 hearts (Fig. 8*F*). These findings suggest that common mechanisms may be involved in the posttranscriptional inhibition of *Clock* translation in both ESCs and E10–12 hearts and that the posttranscriptional regulation of *Clock* may contribute to the emergence of circadian clock oscillation during the later stages of development in mammals.

Although our data suggested that the miRNA genes inhibited the *Clock* 5′ and 3′ UTR-fused luciferase reporter activities, their inhibitory effects were partial and limited. Therefore, we considered the possibility that additional mechanisms contribute to posttranscriptional inhibition of CLOCK expression. By using FISH, we found *Dicer*/*Dgcr8*-dependent nuclear retention of *Clock* transcripts in undifferentiated ESCs (Fig. 8*G* and Dataset S4). Interestingly, the nuclear retention of *Clock* transcripts was dramatically reduced in $Dicer^{-/-}$ and $Dgcr8^{-/-}$ ESCs as well as in the 21-d differentiated ESCs and MEFs that expressed CLOCK protein (Fig. 8*G*). RNA-seq data showed that more reads were mapped to exons than to introns in both ESCs and 28-d differentiated cells, suggesting that the nuclear-accumulated *Clock* transcripts are mainly in their spliced form (Fig. S7*C*). These results suggest that the nuclear retention of *Clock* transcripts may also contribute to the *Dicer*/*Dgcr8*-dependent inhibition of CLOCK protein expression in ESCs in addition to the identified miRNA genes described above.

Taken together, our findings indicate that cell-autonomous circadian rhythms in E10 mouse fetus hearts do not emerge in vivo due to lack of circadian TTFLs, whereas E17 hearts, with the expression of CLOCK protein, exhibit circadian rhythms with their cycling cell-autonomous circadian oscillator (Fig. 8*H*). We revealed that *Dicer*/*Dgcr8*-mediated posttranscriptional regulation of CLOCK contributes to the mechanisms for the initiation of the molecular clock during in vitro cellular differentiation and that posttranscriptional regulation may also be involved in the establishment of circadian TTFLs during ontogenesis.

Discussion

Lack of Cell-Autonomous Circadian Rhythms Around E10 in the Mouse Fetal Heart. Circadian clocks regulate the daily fluctuations of essential biological processes from the molecular to organismal levels

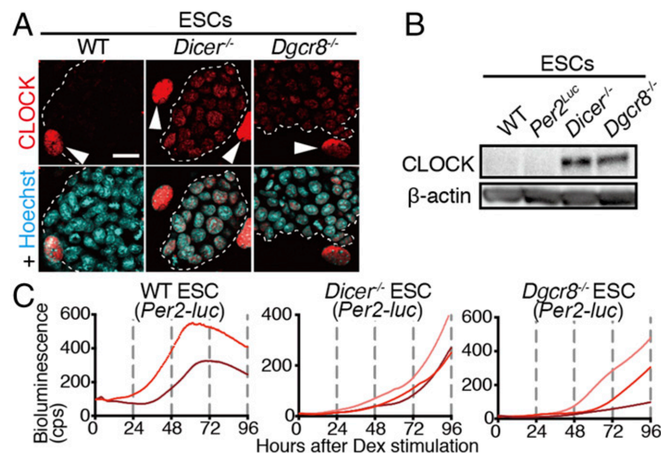

Fig. 7. Absence of circadian clock oscillation in undifferentiated $Dicer^{-/-}$ and $Dgcr8^{-/-}$ ESCs. (*A*) Representative immunostaining of CLOCK proteins in undifferentiated WT, $Dicer^{-/-}$, and $Dgcr8^{-/-}$ ESCs as shown in Fig. 4*A* ($n =$ 2–5 biological replicates). (*B*) Representative Western blots of core CLOCK proteins in WT, $Per2^{Luc}$, $Dicer^{-/-}$, and $Dgcr8^{-/-}$ ESCs ($n = 2$ biological replicates). (*C*) Representative raw bioluminescence traces in undifferentiated WT, $Dicer^{-/-}$, and $Dgcr8^{-/-}$ ESCs carrying *mPer2* promoter-driven luciferase reporters.

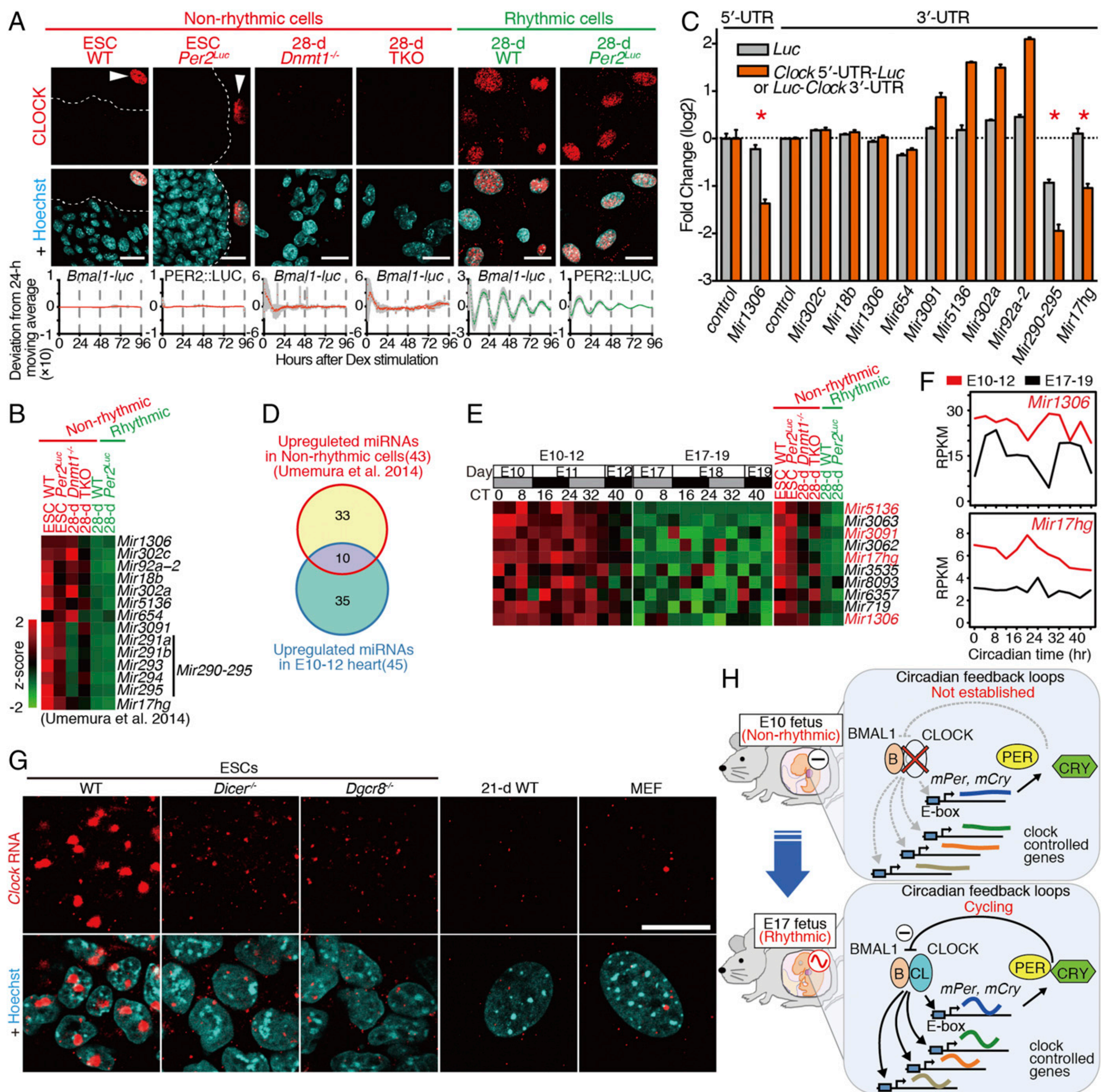

Fig. 8. miRNA-mediated posttranscriptional inhibition of CLOCK protein expression. (*A*) Representative immunostaining of CLOCK in rhythmic and nonrhythmic cells as indicated. The averaged bioluminescence-detrended traces in the indicated cells carrying *Bmal1-luc* reporter (WT, *Dnmt1*$^{-/-}$, and TKO) or *Per2*^{Luc} are shown. Data are shown with the SEM (n = 2–4 biological replicates). (*B*) Heatmap view of the up-regulated miRNA candidate genes targeting *Clock* UTRs in nonrhythmic cells. (*C*) Luciferase reporter assay to validate the miRNA targets. The relative activities of luciferase reporters with *Clock* 5′ UTR (*Clock* 5′-UTR-*Luc*), *Clock* 3′ UTR (*Luc-Clock* 3′-UTR), or no UTRs (*Luc*) were assayed 24 h after cotransfection with the indicated miRNAs or vector plasmid (control). Data are shown with the SEM (n = 3 biological replicates, two-tailed t test, *$P < 0.01$) (*D*) Venn diagram of up-regulated miRNAs in E10–12 hearts and nonrhythmic cells. (*E*) Heatmap view of commonly up-regulated miRNAs in E10–12 hearts and nonrhythmic cells. (*F*) RNA expression levels of *Mir1306* and *Mir17hg* from RNA-seq data. (*G*) Representative image of single-molecule RNA FISH of *Clock* in the indicated ESCs, 21-d differentiated WT ESCs (21-d WT), and MEFs (n = 2 biological replicates). (Scale bar, 20 μm.) (*H*) Model of circadian clock development during gestation. At E10, circadian feedback loops are not yet established in fetal tissues due to posttranscriptional inhibition of CLOCK expression. In the E17 fetus, the CLOCK protein is expressed, the circadian feedback loops are cycling, and the clock-controlled genes are rhythmically expressed and entrained by maternal time cues.

to predict and adapt to the cyclic environment of our rotating planet (44). Cell-autonomous circadian clocks exist in both the SCN and peripheral cells throughout the body (4–7), suggesting that circadian clocks may function as an interface connecting cyclic environmental changes and cellular physiology. Therefore, the emergence of functional circadian rhythms in peripheral cells and SCN neurons is important for mammalian fetal physiology.

We demonstrated that cell-autonomous circadian clock oscillation was undetectable in cardiomyocytes prepared from E9.5

mouse fetal hearts. Moreover, temporal RNA-seq analysis revealed that fewer genes exhibited circadian expression rhythms in E10–12 fetal hearts than in E17–19 fetal hearts in vivo. In mammalian peripheral tissues, both their cell-autonomous oscillator and the temporal cues from the central pacemaker SCN entrain gene expression and functions of peripheral tissues in a circadian manner (45). Similar to peripheral tissues, it is believed that the maternal circadian rhythms entrain the fetus throughout development (15, 16, 29, 46). Supporting the maternal entrainment theory, our RNA-seq data for E17–19 fetal hearts uncovered synchronized rhythms with similar peak phases (Fig. 2*B*). This suggests that global gene expression is strongly entrained by maternal cues in E17–19 embryos. However, the number of fluctuating genes was extraordinarily fewer and the phases of their rhythms were more diverged in E10–12 hearts than in E17–19 hearts (Fig. 2*B*), indicating the possibility that an undeveloped entrainment system responded to maternal circadian cues in E10–12 fetuses. Currently, there is no evidence as to whether the maternal melatonin rhythm can entrain E10–12 mouse fetuses, because the C57BL/6J mouse strain used here lacks melatonin synthesis; this possibility warrants future studies.

Posttranscriptional Regulation of Clock as a Common Mechanism Controlling the Emergence of the Mammalian Circadian Oscillation in ESCs in Vitro and in Developing Hearts in Vivo. Here we demonstrated that the posttranscriptional regulation of *Clock* plays a potential role in the emergence of circadian clock oscillation in mammalian development. As CLOCK protein is switched on in *Dicer*$^{-/-}$ and *Dgcr8*$^{-/-}$ ESCs, DICER/DGCR8 is essential for the posttranscriptional suppression of CLOCK. Furthermore, we identified *Mir1306* and two miRNA clusters (*Mir290–295* and *Mir17hg*) exerting partial inhibitory effects on translational efficiency of *Clock* by targeting the 5′ or 3′ UTR of *Clock* mRNA, which is supported by recent transcriptome analysis in which *Mir294* is proposed as a potential regulator of *Clock* (42). Intriguingly, two of these miRNA genes (*Mir1306* and *Mir17hg*) were also up-regulated in E10–12 mouse fetal hearts in which CLOCK expression was posttranscriptionally inhibited. Furthermore, although the distinct mechanism is not understood, we found that *Dicer*/*Dgcr8*-dependent nuclear accumulation of *Clock* transcripts in undifferentiated ESCs, which is also a possible mechanism controlling the CLOCK expression. It has been reported that cationic amino acid transporter 2 (CAT2) protein expression was inhibited through RNA nuclear retention (47), indicating that *Clock* mRNA retention in the nucleus is a rational reason for posttranscriptional inhibition of CLOCK protein expression. Our study suggests that a posttranscriptional regulatory mechanism contributes to the expression of CLOCK and the emergence of circadian clock oscillation in both mouse fetal tissues and ESCs.

Recently, it was reported that *Npas2*, a paralog of *Clock*, could compensate for *Clock* function in both the SCN and other peripheral cells (48). However, peripheral cells obtained from *Clock*-deficient mice displayed weaker and more unstable circadian molecular oscillation (48), in agreement with previous findings illustrating the importance of *Clock* for circadian rhythms in most peripheral tissues (36). Supporting these results, we demonstrated that CLOCK played a dominant role in the emergence of circadian clock oscillation during in vitro differentiation culture of ESCs. Also, no *Npas2* expression was detected in either ESCs or E10–12 fetal hearts (Fig. 6*D* and Fig. S3*E*), indicating that the repression of *Npas2* expression in undifferentiated cells or in early developmental stages may have additionally contributed to the diminished circadian clock oscillation in these cells.

Meanwhile, a previous report revealed that the cold-induced RNA-binding protein rhythmically regulated CLOCK expression posttranscriptionally in MEFs, which modulate the robustness of circadian clock oscillation (49). Also, it has been reported that *Drosophila Clock* (*Clk*) is regulated posttranscriptionally, which inhibits the ectopic expression of *Clk* and CLK-transcriptional targets (50). Although we could not eliminate the possibility of a *Drosophila*-like mechanism here, these findings suggest that the posttranscriptional regulation of *Clock* may play important roles in establishing and tuning the circadian clock in various species.

Taken together, our findings demonstrated that E10 mouse fetal hearts do not display apparent cell-autonomous circadian clock oscillation, in which the posttranscriptional regulation of *Clock* inhibits CLOCK expression. We showed that similar mechanisms also exist in PSCs such as ESCs. Although the expression of CLOCK on its own is not sufficient for circadian clock oscillation in undifferentiated ESCs, the regulation of CLOCK expression may affect the timing of the emergence of circadian clock oscillation during cellular differentiation and developmental processes in mammals. Therefore, our results suggest that the development of the mammalian circadian clock requires two steps. The first is an epigenetic- and transcriptional program-mediated cellular differentiation process (the cell-lineage determination process), and the second is the establishment of the TTFLs of the mammalian circadian clock in which the posttranscriptional regulation of *Clock* functions as a rate-modulating mechanism (Fig. S8). These sequential mechanisms may explain, at least in part, the late emergence of mammalian circadian clock oscillation in the developmental process.

Materials and Methods

Pregnant C57BL/6J females raised under LD12:12 conditions (lights on at 6:00 AM, lights off at 6:00 PM) were purchased from Japan CLEA, Inc. Animals were maintained under LD12:12 conditions and were transferred to a constant dark condition for 36 h before sampling. The morning after the vaginal plug was found was designated day E0.5. Embryonic heart samples were collected every 4 h for 44 h from CT0 (6:00 AM). The mothers were killed by cervical dislocation in the dark by investigators using night-vision goggles (ATN Night Cougar LT). Then lights were turned on, and embryonic mouse hearts were microdissected in ice-cold PBS (Nacalai Tesque) under a stereomicroscope. The hearts were snap frozen in liquid nitrogen and stored at −80 °C until use. For real-time monitoring of bioluminescence from the embryonic hearts, homozygous *Per2*^{Luc} knockin males (7) were mated with WT C57BL/6J females for one night, and E0.5 was defined as noon of the next day. Pregnant females were maintained under LD12:12 conditions until sampling. All animal experiments were performed in accordance with the guidelines of the Kyoto Prefectural University of Medicine Animal Care and Use Committee.

Detailed methods are provided in *SI Materials and Methods*.

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