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The Circadian Clock Starts Ticking at a Developmentally Early Stage

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Abstract Although overt diurnal rhythms of behavior do not begin until well after birth, molecular studies suggest that the circadian clock may begin much earlier at a cellular level: mouse embryonic fibroblasts, for example, already possess robust clocks. By multiple criteria, we found no circadian clock present in mouse embryonic stem cells. Nevertheless, upon their differentiation into neurons, circadian gene expression was observed. In the first steps along the pathway from ES cells to neurons, a neural precursor cell (NPC) line already showed robust circadian oscillations. Therefore, at a cellular level, the circadian clock likely begins at the very earliest stages of mammalian development.

Key words circadian, differentiation, embryonic stem cell

Circadian rhythms of behavior in many animals are first visible weeks or months after birth. Nevertheless, a large body of evidence suggests that daily biological timekeeping could begin much earlier. For example, in zebrafish, the transcription of the clock gene *per1* begins on the first day of development and is already expressed in rhythmic fashion on the second day when fish are reared in a light-dark cycle (Dekens and Whitmore, 2008). Even a brief pulse of light during the first day of development suffices to synchronize a circadian clock by day 3 (Ziv and Gothilf, 2006). Since zebrafish cells are individually photoreceptive (Whitmore et al., 2000), it is thus likely that circadian environmental signals are immediately and directly transmitted to the developing embryo. In *Drosophila*, which also displays universal circadian photoreception (Plautz et al., 1997), a single pulse of light during the first larval stage immediately after hatching suffices to entrain a circadian rhythm of pupal eclosion several days later (Sehgal et al., 1992). Thus, here too, a circadian clock is functional during the first day of development.

In mammals, when circadian timekeeping begins is unclear. For precocial species like humans, diurnal physiological rhythms like fetal heart rate, respiratory rate, movement, and plasma cortisol can be detected in utero. For altricial species like rats (whose pups are very immature at birth), these diurnal physiological signs are only visible postnatally. At a cellular and tissue level, however, circadian rhythms begin prenatally in both (reviewed in Davis and Reppert, 2001; Seron-Ferre et al., 2001; Sumova et al., 2006).

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The "master clock" of mammals is the suprachiasmatic nucleus (SCN) of the brain hypothalamus. In both classes of mammals mentioned above, circadian rhythms of physiology coincide roughly with the completion of neurogenesis in this nucleus and its innervation by the retinohypothalamic tract, by midgestation in precocial mammals and much later in altricial ones (Seron-Ferre et al., 2001). Notwithstanding, rhythmic daily activity of this nucleus, measured both metabolically and by electrical activity in slice culture, begins fetally in both groups (Reppert and Schwartz, 1983; Shibata and Moore, 1987). One of the most obvious consequences of these fetal clocks is that pups show identical phase to their mother, both in utero and postnatally (Davis and Gorski, 1985). This synchrony is lost if the maternal SCN is ablated, so it is presumed that fetal rhythms are driven or entrained by maternal cues (Davis and Gorski, 1988; Reppert and Schwartz, 1986).

The mechanism of the circadian clock is cell autonomous (Welsh et al., 1995) and present not only in the SCN but in most cells of the body (Yamazaki et al., 2000). Its molecular mechanism is likely based upon interlocked feedback loops of transcription and translation of dedicated "clock gene" loci, including transcriptional activators like *Clock* and *Bmal1* and transcriptional repressors like *Per1*, *Per2*, *Cry1*, *Cry2*, and *Rev-Erbα* (reviewed in Ripperger and Brown, 2009). Direct measurement of clock gene transcripts, as well as the use of clock gene promoter–driven bioluminescent reporters (like *Per1-luciferase*), has permitted the dissection of fetal circadian oscillations at a gross scale. Between E10 and E21, expression of *Per1-luc* in fetal rats measured in one study in vivo steadily increased, with the first evidence of rhythmic expression occurring at E12 (Saxena et al., 2007). Nevertheless, specific analysis of SCN clock gene expression has failed to detect circadian oscillations in the early SCN in multiple cases (Li and Davis, 2005; Sladek et al., 2004). Interestingly, although synchronous circadian oscillations could not be detected in whole mouse embryos or tissues in vivo, they could be detected in some of the same tissues ex vivo, suggesting that a coherent synchronizing signal might be lacking (Dolatshad et al., 2010). Another recent study found rhythmic fetal clock gene expression in the pars tuberalis but not in the SCN, implying that early synchronous circadian oscillations might be the result of maternal signals such as the circadian hormone melatonin to input-driven organs (Ansari et al., 2009).

At a cellular level, the picture is a bit clearer. Although clock transcripts are present in the oocyte, their levels decrease steadily until the 16-cell stage, before rising again at the blastocyst stage (Ko et al., 2000). Multiple studies have shown oscillations of clock or clock reporter gene expression in explanted embryonic fibroblasts, so a cellular clock probably exists as early as E12 (Yagita et al., 2001). Nevertheless, it is unlikely that clocks are present in the earliest embryonic cells: while these studies were underway, work from Yagita et al. (2010) showed that embryonic stem cells do not possess functional circadian oscillators, although cells differentiated from them do. These data suggest that a clock is absent in early pluripotential cell types and rapidly emerges as differentiation begins, even if synchronous circadian oscillations within tissues do not occur until later.

To test this hypothesis explicitly, we investigated circadian oscillations in an embryonic stem (ES) cell line, as well as in a neural precursor cell (NPC) line, and in neurons differentiated from both. Our results suggest that while the circadian clock is indeed silent in primordial stem cells, it is immediately activated in some of the earliest multipotential cells derived from it. Thus, the circadian oscillator "begins to tick" at a cellular level at the very earliest stages of mammalian development.

MATERIALS AND METHODS

Culture and Differentiation of Embryonic Stem Cells

Wild-type mouse embryonic stem cell line E14Tg2A.4 came from Bay Genomics (University of California, Davis). Culture medium for embryonic stem cells consisted of KO-DMEM (Invitrogen, Carlsbad, CA) supplied with 15% FBS Gold (GIBCO, Carlsbad, CA), 2 mM L-glutamine (GIBCO), 0.1 mM nonessential amino acids, 1 U/mL LIF (ESGRO, Millipore, Billerica, MA), and 0.1 mM β-mercaptoethanol (Sigma, St. Louis, MO). Cells were grown on a feeder layer consisting of mitotically incompetent mouse embryonic fibroblasts. Differentiation was achieved by hanging droplet culture of embryoid bodies (EBs) (Conley et al., 2005). After 4 days, individual EBs were differentiated for an additional 4 days in suspension culture in DMEM medium with 4500 g/L glucose (Sigma) containing 10% FBS, 2 mM L-gluthamine, 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, and 5 μM retinoic acid (Sigma). Differentiated embryoid bodies were collected by centrifugation and plated in neurobasal medium (GIBCO) supplemented with

0.5 mM L-glutamine (GIBCO) and 1x B27 supplement (GIBCO, cat. no. 17504-044) on adhesive dishes coated with poly-L-lysine. After 4 to 8 days, the embryoid bodies flattened out, and cells had differentiated to neurons and formed a network on the plate.

Cultivation and Differentiation of Neural Progenitor Cell (NPC) Lines

NPC lines GS-5 and GS-8 derived from human glioblastoma (Gunther et al., 2008) were cultivated in serum-free neurobasal medium (GIBCO) supplemented with 2% B27 supplement (GIBCO, cat. no. 17504-044), 2 mM glutamine (GIBCO), 20 ng/mL human recombinant fibroblast growth factor-2 (PeproTech, Rocky Hill, NJ), 20 ng/mL human recombinant epidermal growth factor (PeproTech), and 32 IU/mL heparin (Sigma). The cultures were incubated at 37 °C with 5% CO_2. Under these culture conditions, the cells grow as free-floating neurospheres. Once weekly, the cells were split by gentle Accutase (Sigma, cat. no. A6964) treatment followed by mechanical dissociation and washing to remove enzyme. The undifferentiated state was maintained by replenishing the growth factors every 2 days. For the differentiation, the neurospheres were collected by sedimentation, washed twice, and plated as neurospheres in 35-mm polylysine-coated dishes in plating medium: neurobasal medium supplemented with 2% B27 supplement (GIBCO, cat. no. 17504-044), 2 mM glutamine (GIBCO), 100 U/mL penicillin/streptomicin (GIBCO), 10% fetal calf serum (Biochrom, Cambridge, UK), and 1 µM retinoic acid (Sigma). This plating favors neurogenesis over gliogenesis (Capowski et al., 2007). The cells were allowed to differentiate for 14 days, with medium change every 3 to 4 days.

Measurement of Circadian Clock Properties in NPC Lines

Infection with lentiviral reporter vectors was carried out as previously described (Brown et al., 2005) with an MOI of 10 at a confluency of 60% for undifferentiated and differentiated neurospheres. There were 500,000 cells in single-cell suspension seeded in a 12-well plate with 1 mL of concentrated virus. After 24 h, the cells appeared aggregated in neurospheres. The virus was removed by centrifugation, and the neurospheres were seeded in 35-mm dishes with neurobasal medium containing B27 supplement, growth factors, and heparin to keep them undifferentiated. The differentiation of infected neurospheres was done as described above. Fourteen days after infection, circadian rhythms of differentiated cells and neurospheres were synchronized with dexamethasone 400 nM for 20 min. After washing, medium without phenol red was supplemented with 0.1 mM luciferin, and circadian rhythms were measured by real-time luminometry (Brown et al., 2005). For neurospheres, the growth factors were present during measurement.

Measurement of Circadian Clock Properties in ES Cell Lines

Embryonic stem (ES) cells and the neurons differentiated from them as well as 3T3 cells were infected with recombinant adenovirus (vector from Invitrogen) containing a *Bmal1-luciferase* cassette identical to our previously described lentiviral construct (Brown et al., 2008) with an MOI of 70 (for 293T cells) for undifferentiated ES cells at 70% confluency as well as 3T3 cells and an MOI of 30 for neurons differentiated from ES cells at 40% to 60% confluency. We estimate that infection efficiency was in the range of 30% to 40%. To measure circadian bioluminescence, infected cells were synchronized with 100 nM dexamethasone for 30 min. Thereafter, the medium was changed to either complete embryonic stem cell medium as described above but supplemented with 0.1 mM luciferin (for ES cells) or the complete medium for stem cell differentiated neurons supplemented with 0.1 mM luciferin. Real-time bioluminescence was measured in a homemade photomultiplier-incubator apparatus at 37 °C, 5% CO_2 (Brown et al., 2005).

Clock Gene Expression Analysis

RNA was extracted as described in *Current Protocols in Molecular Biology* (Kingston et al., 2001). There was 500 ng of total RNA transcribed to cDNA with SuperScript II (Invitrogen) using oligo(dT) primers according to the manufacturer's instructions. For quantitative real-time PCR, 20 ng of cDNA was used, and transcript levels of genes were detected by Taqman probes used with the Taqman PCR mix protocol (Roche, Basel, Switzerland) using the AB7900 thermocycler as described previously (Preitner et al., 2002).

Bioluminescence Time Lapse Microscopy and Data Analysis

ES cells were plated in 35-mm glass bottom dishes (Willco-dish, type 3522, Willco Wells B.V., Amsterdam,

the Netherlands) covered with laminin. After stimulating the cells with 100 nM dexamethasone for 30 min, the medium was replaced by 2 mL phenol red–free DMEM supplemented with 10% FCS and 1 mM luciferin. Bioluminescence imaging was performed on an Olympus LV 200 bioluminescence workstation (Tokyo, Japan) equipped with a 20x UPLSAPO objective (NA 0.75). Cells were kept in a 37 °C chamber equilibrated with humidified air containing 5% CO_2 throughout the microscopy. Bioluminescence emission was detected for several consecutive days using an EM CCD camera (Image EM C9100-13, Hamamatsu, Japan) cooled to –90 °C using exposure times of 30 min. The image series were analyzed employing the ImageJ 1.32 software (National Institutes of Health, Bethesda, MD) (as described in supplementary online materials). To measure the varying bioluminescence response across image sequences from moving cells, we used a slightly adapted version of SpotTracker (Sage et al., 2005), an ImageJ plugin developed by us, as described previously (Dibner et al., 2009).

RESULTS

Embryonic Stem Cells Do Not Have a Functional Circadian Clock

To investigate the circadian clock of mouse ES cells, we developed an adenovirus-based circadian luciferase reporter based upon the promoter of the *Bmal1* gene that was capable of infecting these cells at high titer without affecting their differentiation. After infection, cellular circadian rhythms in these cultures were synchronized with dexamethasone, and bioluminescence was measured during the next 4 days. Cosinor analysis demonstrated no significant rhythmicity (Fig. 1A), whereas equivalent treatment of 3T3 fibroblast cells showed robust diurnal oscillations of reporter expression (data not shown). Equivalent results were obtained using synchronization with a simple medium change containing fresh serum (Fig. 1A, arrow).

From the assays above, it is impossible to determine if the circadian clock as a whole is defective in these cells or if only circadian transcription of our reporter gene is defective, for example, due to lack of a critical promoter-binding factor. We tested this possibility by collecting RNA from dexamethasone-synchronized cultures at regular intervals over 24 h and by analyzing expression levels of a variety of clock genes. No rhythmic oscillation was observed in any gene. Compared to 3T3 cells, expression of some genes (e.g., *Bmal1*, *Per1*, *Per2*) was up to 1000x lower in ES cells, while others were 100x overexpressed (e.g., *Cry1*) or comparably expressed (e.g., *Cry2*, *Rev Erbα*) (Fig. 1B and Suppl. Fig. S1). We concluded that the existence of a functional circadian oscillator analogous to that in adult mammals is unlikely.

Both of the previous methods rely upon populations of cells. Hence, it is formally possible that individual ES cells demonstrate circadian gene expression that is unsynchronized to that of its neighbors. To rule out this hypothesis, we analyzed these cells via low-light microscopy to detect oscillations of bioluminescence in individual cells. No rhythmicity was observed (Fig. 1C and Suppl. Video).

Differentiated ES Cells Show Normal Circadian Oscillations

Although we saw no circadian oscillations in ES cells, we nevertheless expected that circadian oscillations would occur in differentiated tissues. We therefore differentiated our ES cell cultures to attempt to restore circadian oscillations. Using cell aggregation and retinoic acid treatment, we were able to differentiate our ES cells homogenously to neurons, as evidenced by their clear dendritic and axonal outgrowths (Fig. 2A and 2B). Upon synchronization with dexamethasone, these cells showed robust circadian oscillations of *Bmal1-luc* expression (Fig. 2C) of amplitude equivalent to those in 3T3 cells (Suppl. Fig. S2).

Activation of the Circadian Clock Is Developmentally Early

One of the major problems in determining exactly when the circadian clock is activated is that the initial steps of differentiation occur relatively rapidly. For example, in mice, it is clear from numerous publications that embryonic fibroblasts (MEFs) contain a functional circadian oscillator (Yagita et al., 2001), and these cells appear as early as embryonic day 12 (Strutz et al., 1995). Similarly, neurogenesis in most brain regions starts around day 9 and peaks at day 10 to 13 (Finlay and Darlington, 1995). After the initial formation of endoderm and ectoderm from completely pluripotential stem cells, the first step in the differentiation of this lineage is the formation of neural precursor cells (NPCs), a heterogenous population of cells in the embryonic ventricular zone that still divide vigorously but can subsequently differentiate into neurons or astrocytes (Gotz and Sommer, 2005). Various cell line models exist for these NPC cells, all sharing the characteristics of neurosphere formation

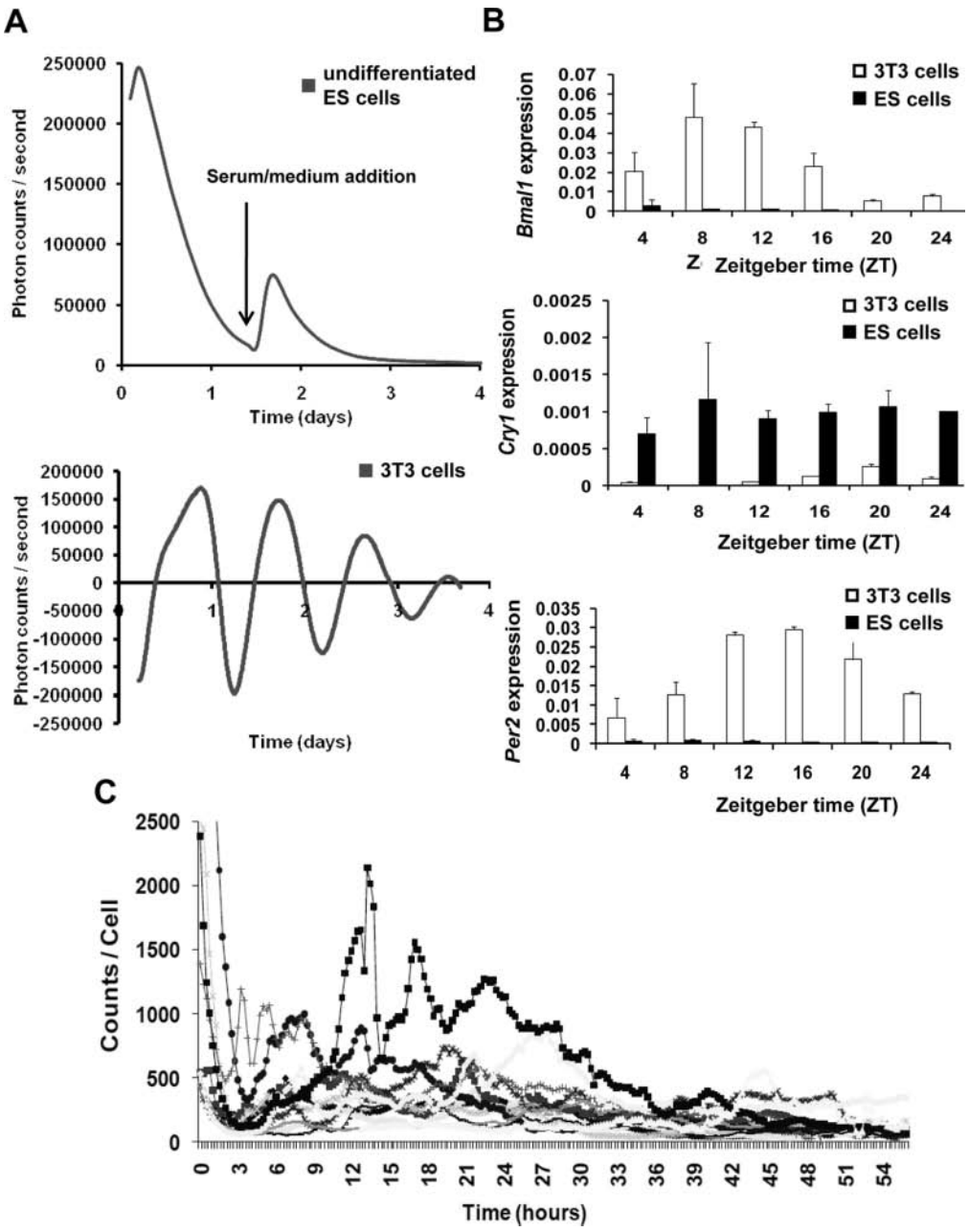

Figure 1. (A) Top panel: bioluminescence from undifferentiated ES cells treated with dexamethasone to synchronize putative circadian oscillations. The arrow is a second synchronization attempt of the same culture using fresh medium + 20% serum; the y-axis is bioluminescence (photon counts per minute); and the x-axis is time in days relative to dexamethasone treatment. Bottom panel: bioluminescence from 3T3 fibroblast cells treated with dexamethasone to synchronize putative circadian oscillations. The y-axis is detrended bioluminescence (absolute photon counts per minute relative to 24-h average of bioluminescence levels); the x-axis is as in A. (B) Expression of *Bmal1*, *Cry1*, and *Per2* gene expression by qPCR from identical plates of undifferentiated ES cells synchronized as in A. In comparison, expression of the same genes in identically synchronized 3T3 fibroblast cells is also shown. The x-axis is hours after synchronization; the y-axis is gene expression expressed as a ratio relative to GAPDH. (C) Bioluminescence traces of individual cells from undifferentiated ES cell cultures synchronized as in A. The y-axis is pixel intensity per cell.

and expression of primordial markers like SOX2, Nestin, DLL3, HES6, NOTCH4, and CD133. Using the cell line GS-5 that expresses all of these (Gunther et al., 2008), we cultivated these cells both as actively dividing neurospheres (believed to represent active NPCs [Svendsen et al., 1998]) (Fig. 3A) and, after treatment with retinoic acid, nondividing and differentiated into neurons (Fig. 3B). Both cultures were infected with reporter virus, synchronized as above, and measured via real-time luminometry. Identical robust rhythms were observed in both cells (Fig. 3C and 3D and Suppl. Fig. S2), implying that the circadian clock already exists at the NPC level. Equivalent results were seen with another line (GS-8, data not shown), but its culture resulted in far greater heterogeneity of cell morphologies.

DISCUSSION

In this article, we showed that the activation of the circadian oscillator occurs during one of the earliest steps of development, at least in the lineage that we examined. Although an embryonic stem cell line showed no functional circadian clocks, a neural precursor cell line and neurons differentiated from both lines showed robust clock activity. Nevertheless, overt rhythms of behavior do not manifest themselves prior to a few weeks of age in mice and 2 months of age in humans (reviewed in Davis

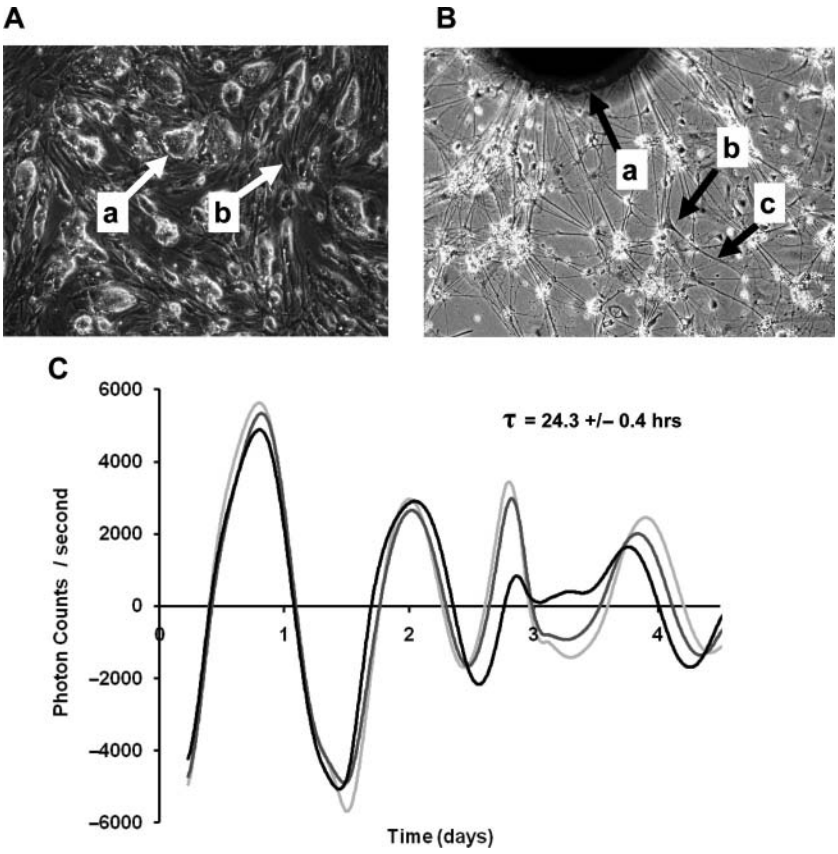

Figure 2. (A) Undifferentiated ES cells grown on a feeder layer of mitotically inactivated fibroblasts. Arrow a = ES cell colony; arrow b = fibroblast. (B) Neurons derived from differentiated ES cells. Arrow a = neuron; arrow b = remains of embryoid body; arrow c = axon. (C) Bioluminescence from cells in B. The x-axis is time in days relative to dexamethasone synchronization; the y-axis is relative bioluminescence (photon counts per second, detrended). Tau is calculated as the average from 3 independent experiments performed in triplicate. Traces are shown from technical triplicates of a single experiment.

and Reppert, 2001; Seron-Ferre et al., 2001). Hence, these cellular rhythms must be desynchronized or suppressed.

One caveat to the interpretation of our results is that we employed stable pluripotential cell lines. These lines may not be identical to the primary cells that they are designed to represent, and they are maintained in culture via a specialized mix of growth factors that prevent them from differentiating. It is possible that these specialized conditions are responsible for the suppression of an otherwise functional circadian clock. We consider this hypothesis unlikely because the same cocktail of growth factors was used to cultivate NPCs (which had clocks) and ESCs (which did not).

Secondly, whether a given cell line represents adequately the same population of cells in vivo is always a valid criticism. For ES cells, in answer, we can say that these cells were subsequently used to generate mice in the course of a different study, verifying their pluripotency. For NPCs, the answer is less clear. Various NPC cell models all suffer from the fact that they are isolated from brain tumors (Svendsen et al., 1998; Tarnok et al., 2010). The principal evidence of their validity as a model system is their expression of primordial cell markers and their ability to be differentiated. We chose the GS-5 line because we could maintain it in culture without traces of differentiated neurons under one set of conditions and differentiate it completely with another and because it expresses numerous primordial markers (Gunther et al., 2008).

The fact that circadian oscillations are not observed in ES cells but can be observed in cells differentiated from them was also previously reported by Yagita et al. (2010). In their study, the proof that ES cells per se possess no normal functional circadian clock was elegantly shown by an additional reverse experiment: dedifferentiation of clock-containing cells back to ES cells resulted in the elimination of functional circadian oscillations (Yagita et al., 2010). They also showed that NPCs possess functional circadian clocks; however, since their NPCs were partially differentiated from their ES cells, the media for these types contained different cocktails of growth factors. Hence, it is possible that the differences that they observed could be driven by external factors. Our own work used identical media for both cell types to eliminate this possibility but instead had the limitation that our NPC cells were cancer derived. The 2 studies together, by examining different ES and NPC lines cultivated under different conditions but assayed by essentially identical methods, make it highly probable that the essential conclusions of both studies are correct.

In normal development, a completely pluripotential ES cell would linger less than a day and a multipotential NPC only 3 to 5 days, making the question of their circadian clock rather academic. More interesting is the

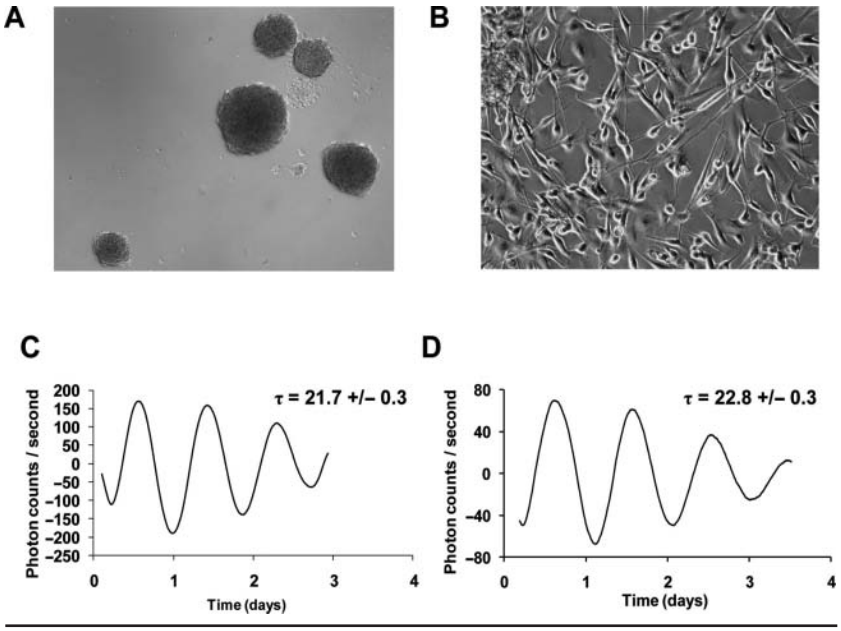

Figure 3. (A) Undifferentiated GS-5 cells grown as neurospheres. (B) Neurons derived from GS-5 cells. (C) Bioluminescence from cells in A. The y-axis is relative bioluminescence (photon counts per second, detrended; the x-axis is time in days relative to dexamethasone synchronization. (D) Bioluminescence from cells in B. Tau is calculated from 2 independent experiments performed in duplicate. Traces are shown from a single representative culture.

question of why a circadian clock is activated so early at a cellular level compared to a behavioral or physiological one. The viability of knockout mice with defective circadian clocks suggests that circadian rhythms in gene expression play no essential role during development. Hence, very early cellular activation of the circadian clock might occur by chance at the same time as other cellular processes, but before the systemic cues necessary for its synchrony and organism-wide manifestation. A second possibility, which we find more interesting, is that a cellular clock itself provides a valuable function even in the absence of intercellular synchrony by segregating mutually harmful processes such as respiration and cell division and thereby confers a selective evolutionary advantage, for example, more effective DNA repair (Collis and Boulton, 2007; Sahar and Sassone-Corsi, 2009).

In any case, it is clear from our results that the circadian clock occurs at a very early time in development. How it is switched on is probably related to the appearance of a full complement of gene products necessary to circadian function, in the ratios necessary for a limit cycle of feedback. Why it is switched on is a question that shall only be answered by much further experimentation.

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NOTE

Supplementary material for this article is available on the journal's Web site: http://jbr.sagepub.com/supplemental.

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