Bioluminescence Imaging of *Period1* Gene Expression In Utero

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Abstract

The use of real-time reporters has accelerated our understanding of gene expression in vivo. This study examined the feasibility of a luciferase-based reporter to image spatiotemporal changes in fetal gene expression in utero. We chose to monitor *Period1* (*Per1*) because it is expressed broadly in the body and plays a role in circadian rhythmicity. Using rats carrying a *Per1::luc* transgene, we repetitively imaged fetuses in utero throughout gestation. We found that bioluminescence was specific to transgenic pups, increased dramatically on embryonic day 10 (10 days after successful mating), and continued to increase logarithmically until birth. Diurnal fluctuations in *Per1* expression were apparent several days prior to birth. These results provide the first in utero imaging of mammalian gene expression, the first tracking of fetal gene expression from the same litter, and the earliest detection of mammalian clock gene expression. We conclude that luciferase-based reporters can provide a sensitive, noninvasive measure of in utero gene expression.

Recent developments in molecular imaging using luciferase-based reporters have provided major insights into normal and pathologic biology in vivo.[1,2] Because luciferase emits dim light in the presence of its substrate, it can be imaged without illumination and avoids photic influences on cell health or physiology. Additionally, because background emission of light by cells (or by luciferase alone) is extremely low, bioluminescence imaging can provide high signal to noise ratios. We reasoned that these qualities could make luciferase-based reporters well suited for monitoring developmental regulation of gene expression in utero.

Circadian rhythms normally entrain to daily environmental cycles and free-run with a period of nearly 24 hours in the absence of environmental cues. In animals, rhythm generation is believed to depend on transcriptional regulation of key "clock" genes, including homologues of the *Period* (*Per*) gene. In mammals, *Per1* messenger ribonucleic acid (mRNA) and protein oscillate in many cell types.[3,4] Results from lesion, transplant, and in vitro experiments strongly implicate the suprachiasmatic nucleus (SCN) as the master pacemaker for circadian rhythms in the brain and body. It is, however, unclear how circadian periodicity is initiated during development.

Mammalian circadian rhythmicity likely develops in utero. Humans express rhythms in heart rate, blood pressure, body temperature, and cortisol on the day of birth.[5,6] Day/night differences in 2-deoxyglucose (2DG) uptake indicate metabolic rhythms in the fetal primate SCN.[7] Neurogenesis in the rat SCN occurs from embryonic day (E)14 to E17 (where E1 is the day after successful mating),[8] and day/night differences in 2DG uptake have been detected as early as E20 days.[9] (Embryonic ages in this article are reported as days after mating according to the convention of Altman and Bayer.[10]) *Per1* mRNA is rhythmically expressed by E17 in the mouse SCN[11] and by E21 in the rat SCN.[12] Whether this marks the initiation of circadian regulation in the brain and body is unknown.

Previous studies indicated that circadian rhythms are intrinsic to the fetal SCN. In vitro experiments

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demonstrate that firing rates in the E22 rat SCN are higher during the subjective day than at night.[13] Additionally, E17 hypothalamic tissue can restore locomotor rhythms to SCN-lesioned adults,[14] and prenatal exposure to maternal signals, melatonin or dopamine, affects the circadian phasing of pups around the time of weaning.[15,16] Destroying the maternal SCN abolishes the SCN-2DG rhythm within a litter but not the postnatal circadian locomotor patterns of individual pups.[17,18] These data led to the hypothesis that the maternal SCN entrains fetal circadian rhythms but is not required for their generation. Efforts to establish when and where circadian cycling initiates, however, have been complicated by available techniques. Because results were pooled from tissues derived from litters fixed and processed at different times, it was impossible to discern whether arrhythmicity resulted from a lack of pacemaker function or a lack of synchrony among the pups.

We used transgenic rats in which a firefly luciferase transgene is driven by the *Per1* gene promoter *(Per1::luc*[19]) to examine the ontogeny of *Per1* expression in utero.

Materials and Methods

Animals

Japanese Wistar rats were maintained in a 12 hours of light, 12 hours of dark schedule (lights on at 0700) in the Danforth Animal Facility at Washington University unless otherwise noted. For timed pregnancies, proestrous females at least 3 months old were paired with males overnight. Within 3 hours after lights on the next morning, impregnation was confirmed on visualization of a copulatory plug. E1 and postnatal day (P)1 were defined as the day after successful mating and day of birth, respectively. All procedures were approved by Washington University IACUC and conformed to US National Institutes of Health guidelines. 2

In Vivo Imaging of *Per1::luc* Expression

Wild-type females were mated to heterozygous *Per1::luc* transgenic males. For three independent litters, dams were imaged daily within 1 hour of light onset or offset starting on E7. D-Luciferin (150 mg/kg body weight; Xenogen) was injected intraperitoneally after the females were briefly anesthetized (2.5–3% isofluorane vaporized in 1 L/min O_2). Five minutes postinjection, dams were reanesthetized, and their shaved ventral surface was imaged beginning 10 minutes postinjection at five different exposure times (120, 60, 30, 10, and 1 second) using an IVIS Imaging System and a charge-coupled device camera (15 cm field of view, binning 8, 1/f stop, open filter; 100 series; Xenogen). Neonates were visualized using the same protocol but injected with 1.5 mg/kg of D-luciferin and imaged in a 20 cm field of view. In utero bioluminescence from the abdomens of dams was analyzed using *Living Image* 2.20 (Xenogen) and *Igor* (Wavemetrics) software. Total photon flux (photons/s) was measured from the longest, non-saturating exposure at each time point.[20] 3 4 5

Statistical Analysis

To determine the earliest time when *Per1* expression of the pups could be detected, we considered the dawn and dusk bioluminescence from the abdomen of each dam to represent repeated measurements. We could not visually detect any bioluminescence signal over background until E9, nor could we detect bioluminescence signals over background postpartum. We, therefore, considered daily repeat measurements of the same rats prior to E9 ($n = 4$ for rat 1, 6 for rat 2, and 14 for rat 3) and on P4 ($n = 2$ for rats 1 and 3; rat 2 died in delivery) as independent measurements of background activity.

Repeated measurement analysis[21] was performed on each dam and used to calculate the difference between the original (dawn) and repeated (dusk) measurement. This difference was plotted against the mean of the corresponding dawn and dusk measurements.[22] We excluded one dawn-dusk pair of measurements (from rat 3, day 6) as artifactual because it was 30 SD above the mean dawn-dusk difference of all other measurements. We calculated the 95% confidence intervals (CIs) for the upper and lower limits of agreement (lower limit of agreement between dawn and dusk: −515,827 p/s, 95% CI of −762,900 to −268,755; upper limit: 397,289, 95% CI of 150,216 to 644,361). Measurements exceeding background variation in bioluminescence by greater than the 95% CI (ie, > 644,361 p/s) were considered significant.

Results and Discussion

Per1 Expression In Utero

We mated wild-type dams with heterozygous transgenic males expressing firefly luciferase under the control of the mouse *Per1* promoter (*Per1::luc*).[19] We recorded high levels of reporter expression in heterozygous pups during gestation (Figure 1; $n = 3$ litters) and after birth (Figure 2). Images of neonates revealed light emission from the skin

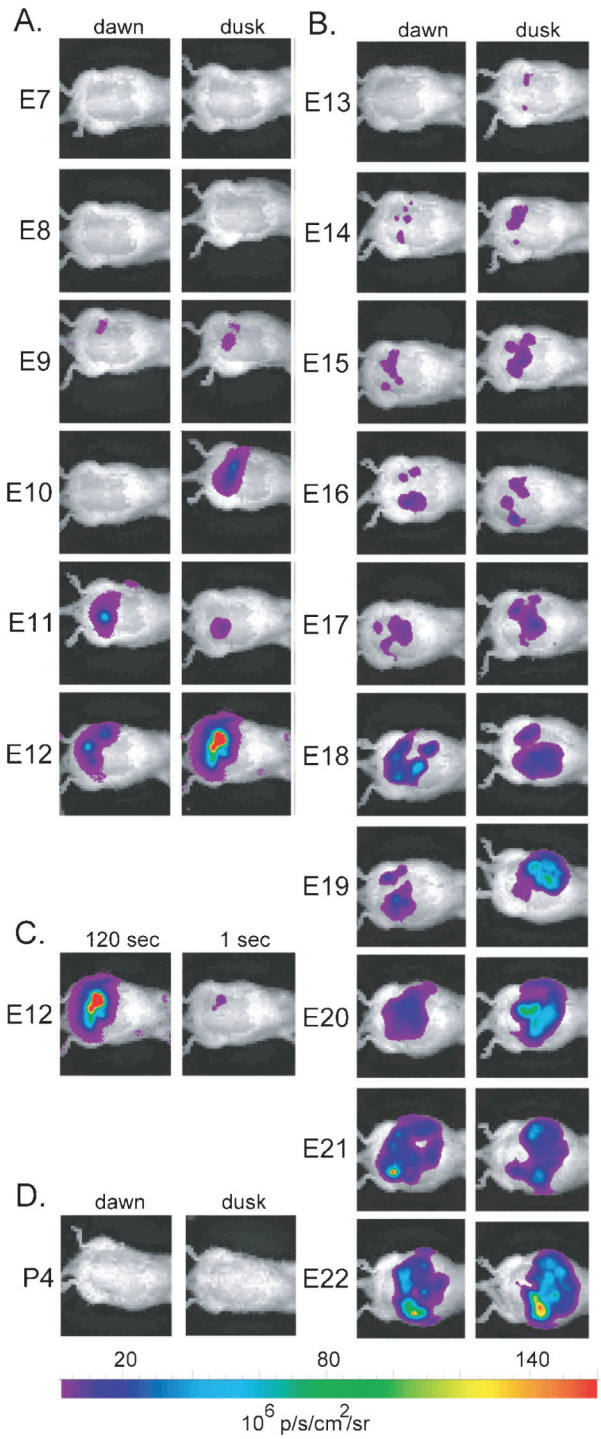

Figure 1. Bioluminescence from transgenic fetuses imaged in utero throughout gestation of a wild-type female rat. Photon flux (p/s/cm^2/sr) from the heterozygous *Per1::luc* embryos is displayed according to the scale bar at the bottom. *A*, Daily 120-second exposures at dawn and dusk show that light emission was detectable by emybryonic day (E)9 and increased dramatically on the evening of E10. *B*, Dawn and dusk exposures (1 second) from E13 to E22. Individual pups are discernible in many of the images but did not remain in the same position throughout gestation. *C*, After E12, exposures longer than 1 second tended to saturate the camera. For comparison, light emission from the same female is shown on E12 after integrating for 120 seconds and 1 second. *D*, Dawn and dusk images of a dam 4 days after delivery. Dams did not bioluminesce after pups were born (postnatal day 4; 120-second exposure).

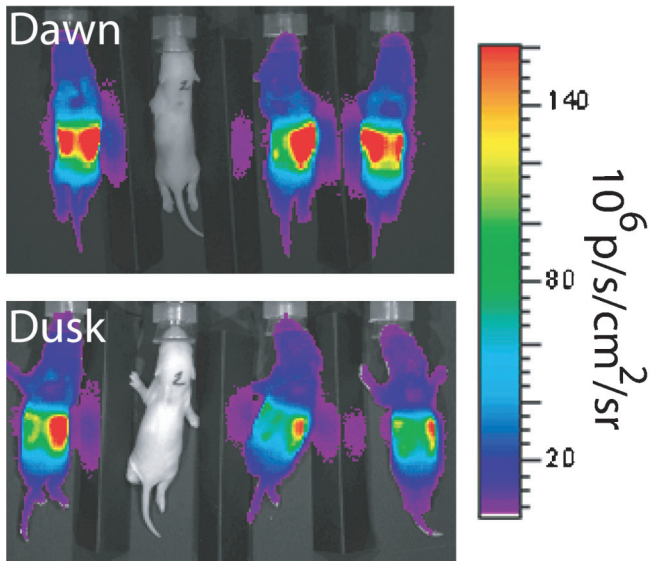

Figure 2. Transgenic pups bioluminesce, whereas wild-type littermates (second from the left in each image) do not. Representative 1-second dawn and dusk images on postnatal day 4 show that the majority of the signal comes from the neonatal liver.

and internal organs, especially the liver. This is consistent with the broad expression patterns of *Per1*.[19,23–30] In contrast, nontransgenic littermate pups, dams 4 days postpartum, and nonpregnant adult females did not bioluminesce (see Figures 1D and 2 and data not shown). Thus, *Per1* promoter-driven bioluminescence functions as a specific reporter of transgene activity in heterozygous fetuses.

Average bioluminescence in utero increased logarithmically from E10 to E22 (Figure 3). Pups from the three dams consistently exceeded background bioluminescence after E2, E7, or E12, respectively (see Methods for statistics). We found that E12 fetuses ($n = 4$) emitted light 760 times background on average when measured in vitro over 48 hours using a photomultiplier tube and published methods.[31] Notably, the fetal SCN begins to form on E14, several days after detectable *Per1* expression. Dawn-dusk fluctuations were apparent on some embryonic days but not all days for the three litters. For example, average litter bioluminescence was higher around dusk on E19, E20, and E22, consistent with the timing of *Per1* expression in the adult rat SCN.[19] Factors including developmental changes in *Per1* expression in multiple and

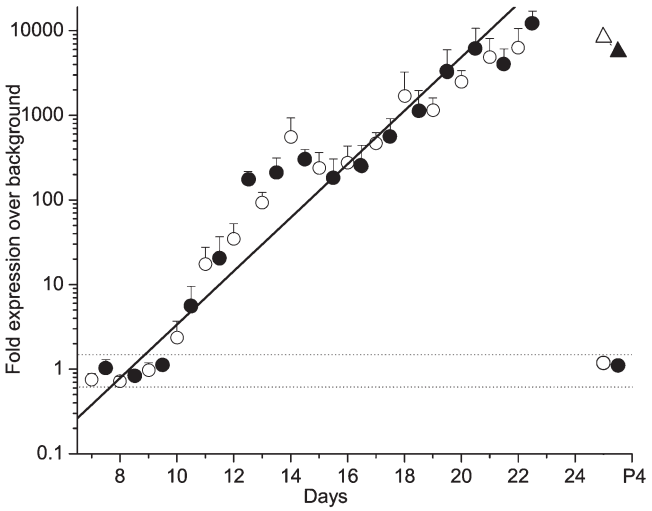

Figure 3. Mean total photon flux from pregnant and postpartum females (*circles*, $n = 3$) and postnatal day 4 pups (*triangles*, $n = 3$) at dawn (*white*) and dusk (*black*) plotted as fold over background. *Per1* activity in the heterozygous fetuses increased well above background (range shown by *dashed lines*) starting at embryonic day (E)10, with a suggestion of day-night fluctuation observed late in gestation (E19, E20, E22). The log-plotted data were well fit by a linear function (*solid line*; $y = 0.32 \times -2.6$; $r = .96$, $p < .0001$). Error bars represent the standard error of measurement.

diverse tissues and the movement of the pups may have confounded the reliable measurement of circadian rhythms, especially during the final days of gestation. Regardless, these results provide compelling evidence for *Per1* expression prior to SCN neurogenesis and suggest the potential for future experiments aimed at determining when or where circadian expression initiates.

Evaluation of Luciferase for In Utero Imaging

The present results establish a relatively noninvasive method for monitoring gene expression in utero. Firefly luciferase-D-luciferin is currently uniquely positioned to image in utero gene expression in animal models, in part because of the short half-life of the reporter. We found that D-luciferin readily crossed the blood-placenta barrier. Other techniques with selected injectable fluorophores or radiotracers (eg, positron emission tomography or single-photon emission computed tomography use of HSV-TK or SSTR-2) have not yet been employed for in utero imaging. Importantly, the cognate radioligands (eg, ^{18}F-FHBG and ^{111}In octreotide) have been shown not to cross the blood-brain barrier and are unlikely to cross the blood-placenta barrier. We were able to make measurements twice daily without disrupting normal development or delivery. Higher temporal resolution is theoretically possible, although the half-life of firefly luciferase protein (approximately 6 hours at 37°C[20]) and the tolerance of dams to repeated anesthesia will likely impose lower bounds. The bioluminescent signal provided tissue-level resolution such that signals from individual fetuses and, in some cases, their livers were readily discernable. Resolution of individual pups was facilitated by reducing the number of glowing fetuses in utero to the heterozygous offspring produced from crossing wild-type females to *Per1::luc* heterozygous males.

A major advantage of this noninvasive reporter system is the ability to follow the same litter through gestation. Previous studies have examined *Per1* expression later in gestation by in situ hybridization. Although circadian rhythms have been detected in the fetal mouse SCN approximately 3 days prior to birth,[11] studies on the rat and hamster SCN failed to find circadian rhythms before the day prior to birth.[12,32,33] The absence of clock gene rhythms has not been reconciled with the reported day-night differences in metabolism reported in the fetal SCN. Based on repeated measurement analysis of bioluminescence of pups, we estimated the earliest detectable expression of *Per1* in utero from three dams to be between days 2 and 12. Future studies designed to include repeated measurements from nonexpressing tissues will better define background levels and improve the precise determination of earliest detection dates and criteria for day-night changes.

We conclude that the combination of in utero and in vitro bioluminescence reporter measurements holds promise as a method for establishing, with high temporal and spatial resolution, when and where dynamic gene regulation begins (eg, circadian modulation in utero of *Per1*).

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References

1. Contag CH, Bachmann MH. Advances in in vivo bioluminescence imaging of gene expression. Annu Rev Biomed Eng 2002;4:235–60.
2. Gross S, Piwnica-Worms D. Spying on cancer: molecular imaging in vivo with genetically encoded reporters. Cancer Cell 2005;7:5–15.
3. Reppert SM, Weaver DR. Molecular analysis of mammalian circadian rhythms. Annu Rev Physiol 2001;63:647–76.

4. Zheng B, Albrecht U, Kaasik K, et al. Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. Cell 2001;105:683–94.
5. Seron-Ferre M, Riffo R, Valenzuela GJ, Germain AM. Twenty-four-hour pattern of cortisol in the human fetus at term. Am J Obstet Gynecol 2001;184:1278–83.
6. Sitka U, Weinert D, Berle K, et al. Investigations of the rhythmic function of heart rate, blood pressure and temperature in neonates. Eur J Pediatr 1994;153:117–22.
7. Reppert SM, Schwartz WJ. Functional activity of the suprachiasmatic nuclei in the fetal primate. Neurosci Lett 1984;46:145–9.
8. Moore RY. Development of the suprachiasmatic nucleus. In: Klein DC, Moore RY, Reppert SM, editors. Suprachiasmatic nucleus: the mind's clock. New York: Oxford University Press; 1991. p. 391–404.
9. Reppert SM, Weaver DR. The biological clock is oscillating in fetal suprachiasmatic nuclei. In: Klein DC, Moore RY, Reppert SM, editors. Suprachiasmatic nucleus: the mind's clock. New York: Oxford University Press; 1991. p. 405–18.
10. Altman J, Bayer SA. The development of the rat hypothalamus. Adv Anat Embryol Cell Biol 1986;100:1–178.
11. Shimomura H, Moriya T, Sudo M, et al. Differential daily expression of Per1 and Per2 mRNA in the suprachiasmatic nucleus of fetal and early postnatal mice. Eur J Neurosci 2001;13:687–93.
12. Ohta H, Honma S, Abe H, Honma K. Effects of nursing mothers on rPer1 and rPer2 circadian expressions in the neonatal rat suprachiasmatic nuclei vary with developmental stage. Eur J Neurosci 2002;15:1953–60.
13. Shibata S, Moore RY. Development of neuronal activity in the rat suprachiasmatic nucleus. Brain Res 1987;431:311–5.
14. Aguilar-Roblero R, Garcia-Hernandez F, Aguilar R, et al. Suprachiasmatic nucleus transplants function as an endogenous oscillator only in constant darkness. Neurosci Lett 1986;69:47–52.
15. Viswanathan N, Davis FC. Single prenatal injections of melatonin or the D1-dopamine receptor agonist SKF 38393 to pregnant hamsters sets the offsprings' circadian rhythms to phases 180 degrees apart. J Comp Physiol [A] 1997;180:339–46.
16. Davis FC, Gorski RA. Development of hamster circadian rhythms: prenatal entrainment of the pacemaker. J Biol Rhythms 1985;1:77–89.
17. Shibata S, Moore RY. Development of a fetal circadian rhythm after disruption of the maternal circadian system. Brain Res 1988; 469:313–7.
18. Weaver DR, Reppert SM. Periodic feeding of SCN-lesioned pregnant rats entrains the fetal biological clock. Brain Res Dev Brain Res 1989;46:291–6.
19. Yamazaki S, Numano R, Abe M, et al. Resetting central and peripheral circadian oscillators in transgenic rats. Science 2000;288: 682–5.
20. Luker GD, Pica CM, Song J, et al. Imaging 26S proteasome activity and inhibition in living mice. Nat Med 2003;9:969–73.
21. Gluer CC, Blake G, Lu Y, et al. Accurate assessment of precision errors: how to measure the reproducibility of bone densitometry techniques. Osteoporos Int 1995;5:262–70.
22. Bland JM, Altman DG. Measuring agreement in method comparison studies. Stat Methods Med Res 2006;8:135–60.
23. Sun ZS, Albrecht U, Zhuchenko O, et al. RIGUI, a putative mammalian ortholog of the Drosophila period gene. Cell 1997;90: 1003–11.
24. Takumi T, Matsubara C, Shigeyoshi Y, et al. A new mammalian period gene predominantly expressed in the suprachiasmatic nucleus. Genes Cells 1998;3:167–76.
25. Tei H, Okamura H, Shigeyoshi Y, et al. Circadian oscillation of a mammalian homologue of the Drosophila period gene. Nature 1997;389:512–6.
26. Shigeyoshi Y, Taguchi K, Yamamoto S, et al. Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript. Cell 1997;91:1043–53.
27. Masubuchi S, Honma S, Abe H, et al. Clock genes outside the suprachiasmatic nucleus involved in manifestation of locomotor activity rhythm in rats. Eur J Neurosci 2000;12:4206–14.
28. Wilsbacher LD, Yamazaki S, Herzog ED, et al. Photic and circadian expression of luciferase in mPeriod1-luc transgenic mice in vivo. Proc Natl Acad Sci U S A 2002;99:489–94.
29. Kuhlman SJ, Quintero JE, McMahon DG. GFP fluorescence reports Period 1 circadian gene regulation in the mammalian biological clock. Neuroreport 2000;11:1479–82.
30. Yamaguchi S, Mitsui S, Miyake S, et al. The 5′ upstream region of mPer1 gene contains two promoters and is responsible for circadian oscillation. Curr Biol 2000;10:873–6.
31. Abe M, Herzog ED, Yamazaki S, et al. Circadian rhythms in isolated brain regions. J Neurosci 2002;22:350–6.
32. Sladek M, Sumova A, Kovacikova Z, et al. Insight into molecular core clock mechanism of embryonic and early postnatal rat suprachiasmatic nucleus. Proc Natl Acad Sci U S A 2004. [8]
33. Li X, Davis FC. Developmental expression of clock genes in the Syrian hamster. Brain Res Dev Brain Res 2005. [9]

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