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Clock controls timing of mouse pancreatic differentiation through regulation of Wnt- and Notch-based and cell division components

Zhixing Li [a,b,*], Lingjuan Ruan [a], Shuibin Lin [a], George K. Gittes [c]

[a] *Department of Biomedical Science, School of Life Sciences, Xiamen University, Xiamen, Fujian 361005, PR China*
[b] *Department of Surgical Research, Children's Mercy Hospital, Kansas City, 2401 Gillham Road, MO 64108, USA*
[c] *Department of Pediatric Surgery, Children's Hospital of Pittsburgh, 3705 Fifth Avenue, Pittsburgh, PA 15213, USA*

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Abstract

The oscillations of circadian genes control the daily circadian clock, regulating a diverse array of physiologies with the 24-hour light/dark cue across a wide variety of organisms. Here we first show that before embryonic circadian rhythms occur, the oscillation (nucleocytoplasmic shuttling) of core circadian gene Clock is tissue-specific and correlated with the state of differentiation during both early development and later pancreas organogenesis. Disruption of Clock as well as Timeless in the embryonic pancreas does not block pancreatic differentiation but alters the balance and maturity of endocrine and exocrine cells. Molecular analysis indicates that inhibition of Clock or Timeless expression disturbs not only cell cycle regulators, but also Wnt- and Notch-signaling components, whose oscillations establish the timing mechanism in somitogenesis. Thus, our results provide new insights about circadian genes' function in control of the timing of differentiation during embryonic development.

Keywords: Clock; Timeless; Embryogenesis; Pancreas development

Daily physiological rhythms are governed by an internal Clock made up of molecular auto regulatory feedback loops entrained by the external 24-hour light/dark cue. The molecular timing mechanism for circadian rhythms turns out to be conserved throughout evolution. In mammals the timing device is driven by Clock/Bmal1 expression [1] and closed by the feedback of several interlocked loops, in which Per1-3 [2], Cry1-2 [3], Dec1-2 [4] and Tim [5] play a central role.

Core circadian genes such as Timeless and Clock have been found to play important roles in many biological activities. Clock mutation in the mice severely alters the diurnal rhythm in food intake and the expression of several key molecules governing metabolism, and results in obesity and metabolic syndromes [6]. Intriguingly, homozygous deletion of Clock locus in both mouse [7] and *Drosophila* [8] is embryonic lethal. Thus, the Clock gene might play some role in the embryonic development.

The progression of embryonic development is apparently orchestrated by a largely unknown internal timing mechanism. Whether or not a timing device similar to that of circadian Clock exists during mouse embryonic differentiation is at question. However, in *Drosophila*, a maternal enhancer gene Vrille, which was originally found to be essential for embryonic development, turned out also to be a core circadian gene [9,10]. Importantly, in Per mutant flies, short or long circadian cycles have resulted in faster or slower embryonic development respectively. Similarly, in plants, circadian genes have been shown to regulate flowering, a developmental process [11]. Moreover, a *C. elegans* protein LIN-42, a PER homolog, controls several developmental events and oscillates with a period corresponding to its molt development cycle [12]. Although we had identified

* Corresponding author. Address: Department of Biomedical Science, School of Life Sciences, Xiamen University, Xiamen, Fujian 361005, PR China. Fax: +86 592 2181015.
E-mail address: zli@xmu.edu.cn (Z. Li).

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the mammalian Timeless (Tim) to be important for kidney branching tubulogenesis [13], whether Timeless had a role in maintaining circadian rhythms was in dispute [14–17]. So we decided to focus on Clock [18,19], the undisputable circadian core element, during mouse embryogenesis.

Here we report evidence that throughout embryonic development, the core circadian genes exemplified by Clock are expressed coordinately with tissue differentiation and could serve as time-keeping device through the regulation of Wnt- and Notch-based components as well as cell cycle regulators.

Materials and methods

Tissues and materials. CD1 mice (Charles River Laboratory) were maintained under normal 12-hour light/dark conditions. At noon of the day of the appearance of vaginal plug was designated as E0.5. Pregnant mice were sacrificed at early morning for antisense treatments or around noon time for immunohistostainings. Embryos and tissues obtained from pregnant CD1 mice were fixed in 4% formalin for 2 h and frozen for 7 μm sections.

Pancreas explant culture. Early E11 (37–42 somites) mouse embryonic pancreas was collected, dissected in half and recombined with each other together when positioned in 3-D collagen gels (one in the antisense condition and the other one in control conditions) which was solidified in inserts and placed in 24-well plates. Tissue was cultured ($n = 3$–6) in triplicate in DMEM/F-12K medium with 10% FBS for 6 days in the presence of 40 μM of Clock or Timeless antisense, sense or scramble morpholino-ring modified oligonucleotides or blank (medium alone) before cultured tissues were harvested for RT-PCR and immunohistostaining analysis.

Immunohistostaining. The following primary antibodies were used: rabbit Clock polyclonal (Affinity BioReagents); Guinea Pig anti insulin polyclonal (DAKO); rabbit anti amylase polyclonal (Sigma); mouse anti glucagon monoclonal (Sigma). Secondary antibodies were purchased from Jackson ImmunoResearch.

RT-PCR analysis. To ensure specificity in the amplification, hot-start polymerase (AmpliTaq Gold, Applied Biosystems) was used in the following "Touch Down" program: 94 °C, 7 min; 94 °C, 40 s, 72 °C, 40 s, 5 cycles; 94 °C, 40 s, 70 °C, 45 s, 5 cycles; 94 °C, 40 s, 68 °C, 50 s, 25 cycles; 72 °C, 7 min; 4 °C. Nested PCR are used and the reaction conditions were the same as the 1st round amplification.

Results

Nucleocytoplasmic shuttling of Clock correlates with tissue differentiation

We first examined the spatial activity of Clock expression during embryogenesis. Immunohistostaining revealed that Clock was expressed shortly after implantation (Fig. 1A and B). Interestingly, around this stage (E5–E6), Clock was found to be localized to the nucleus in the visceral endoderm and other extra-embryonic tissues, while

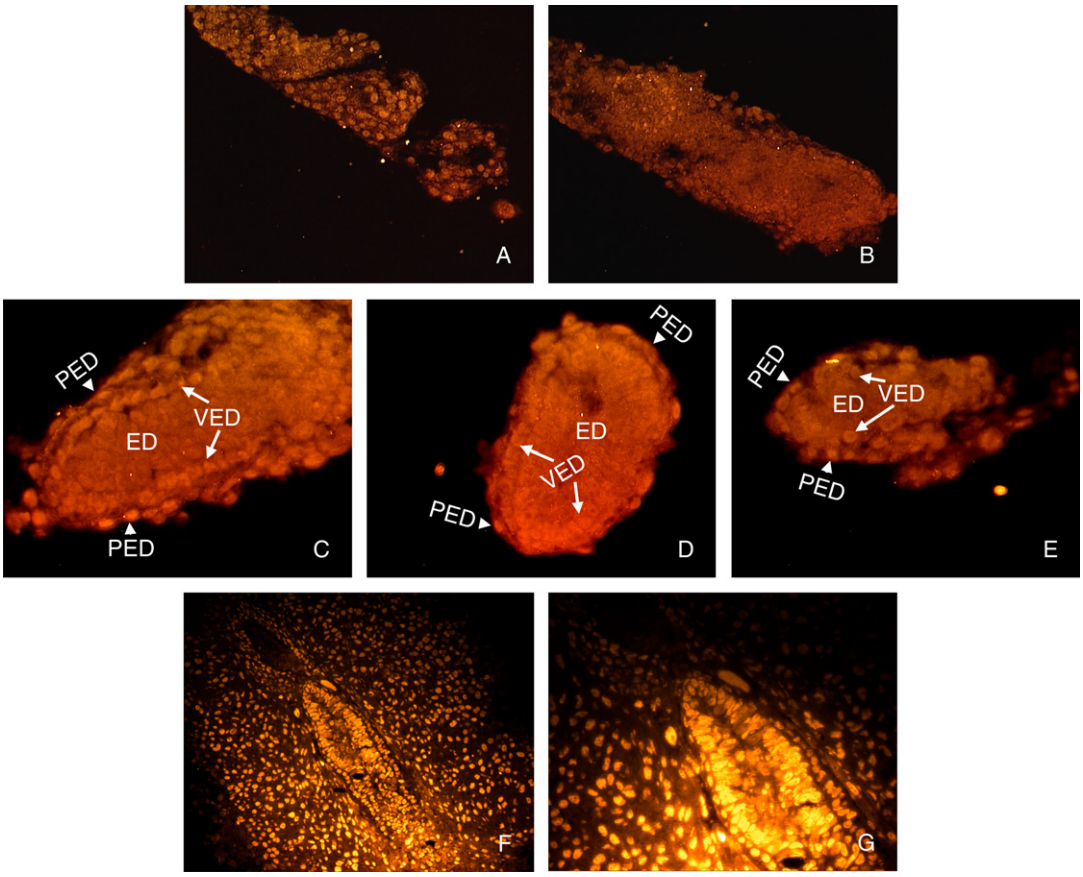

Fig. 1. Clock detection during early embryogenesis. A–F are arranged correspondently with the progression of embryogenesis: (A) E4.5-E5, note nuclear localization; (B) early E5; (C–E) E5.5; (F) early E6. (G) is high power image of (F). ED, ectoderm; VED (arrow), visceral endoderm; PED (arrowhead), parietal endoderm.

it remained in the cytoplasm in embryonic ectoderm (Fig. 1C–E). In subsequent stages, Clock was seen largely in the nuclei until the mid- and late-gestation period (Fig. 1F and G). Notably, similar to the pattern of Timeless expression, Clock expression in extra-embryonic tissues (especially the ectoplacental cone) was often different from embryo proper in terms of nuclear-cytoplasmic localization (Fig. 1). This tissue-specific and differentiation-correlated expression, long before any embryonic physiological rhythms occur, suggests possible Clock's involvement in developmental processes.

We also detected the oscillation of Clock expression during later pancreas organogenesis. At early E13, Clock was found mainly in the nucleus in the pancreatic anlagen, i.e., in both developing epithelium and mesenchyme (Fig. 2A). As the epithelium further developed, Clock started to become more cytoplasmic in some epithelial cells (Fig. 2A, arrowhead). In slightly later (in embryos from the same litter that were a few hours older based on embryonic staging criteria) E13.5 pancreas, Clock remained in the nuclei in mesenchyme, but had become cytoplasmic in most of the pancreatic epithelial cells (Fig. 2B). At E15 pancreas, the nuclear localization in the mesenchyme and nucleocytoplasmic shuttling in the epithelium of Clock expression persisted (Fig. 2C and D). Thus, the nucleocytoplasmic shuttling of Clock coincides with stages of tissue differentiation apparently independent of possible circadian cues per se. Our data suggest that circadian genes take part in mouse embryonic tissue differentiation.

Inhibition of Clock or Timeless alters the degrees of pancreatic endocrine and exocrine differentiation

To further test Clock's function in pancreas development, we made use of an in vitro 3-D embryonic pancreas culture system [20], which simulates the delicate and easily detectable pancreatic endocrine/exocrine differentiation. Inhibition of Clock expression with morpholino ring-modified antisense oligonucleotides in the 3-D pancreas culture for 6 days did not block either endocrine (insulin and glucagon) or exocrine (amylase) differentiation. Instead, the inhibition seemed to alter the pace of differentiation exemplified by the change of balance and maturity of the endocrine/exocrine expression (Fig. 3). We further expanded this functional test to Timeless. Inhibition of Tim also altered the pace of endocrine/exocrine differentiation, although in a manner different from that of Clock inhibition (Fig. 3): i.e., Clock inhibition appeared to accelerate both endocrine (insulin and glucagons) and exocrine (amylase) differentiation while Timeless inhibition accelerated β-cell (insulin) but decelerated acinular cell with little effect on α-cell differentiation. Moreover, the size of the 6-day-culture, antisense-treated tissues also seemed to be different from that of control cultures. Considering the fact that the

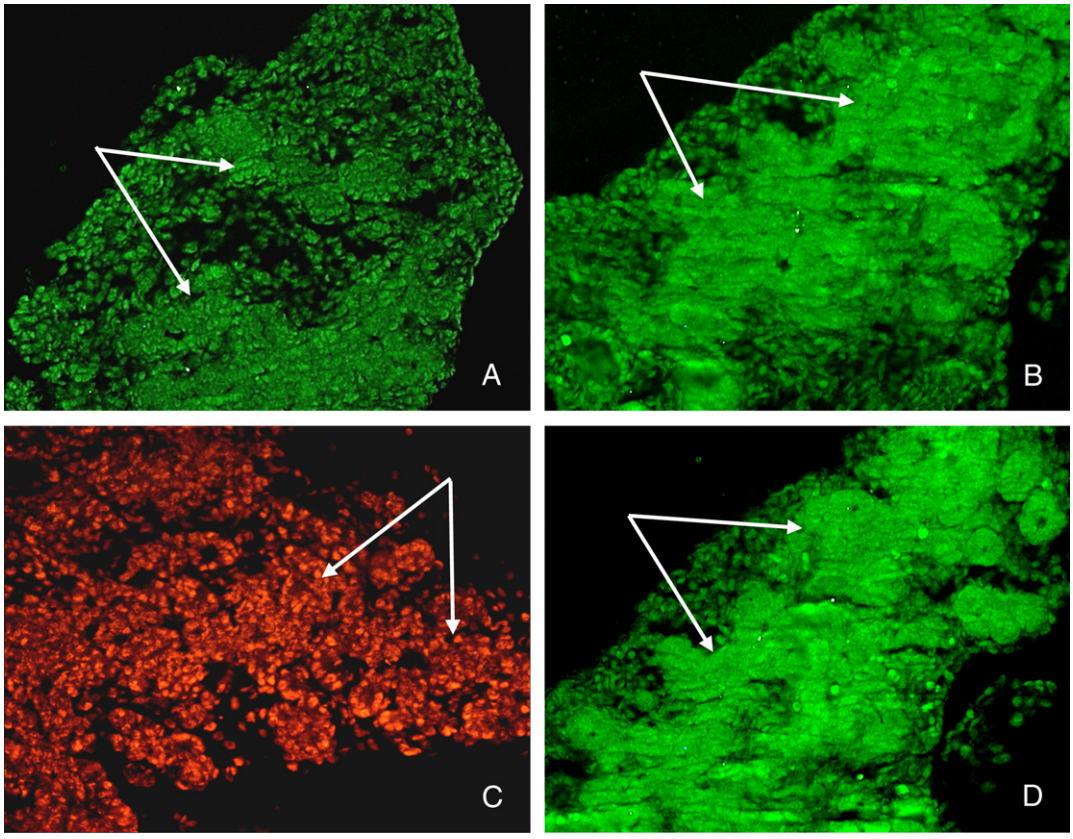

Fig. 2. Clock expression during pancreatic differentiation. (A, C) Earlier E13 and E15 pancreas sections, respectively (arrows show epithelial regions with cytoplasmic expression). Correspondingly, (B and D) are slightly older (littermate) E13 and E15 pancreas sections, respectively.

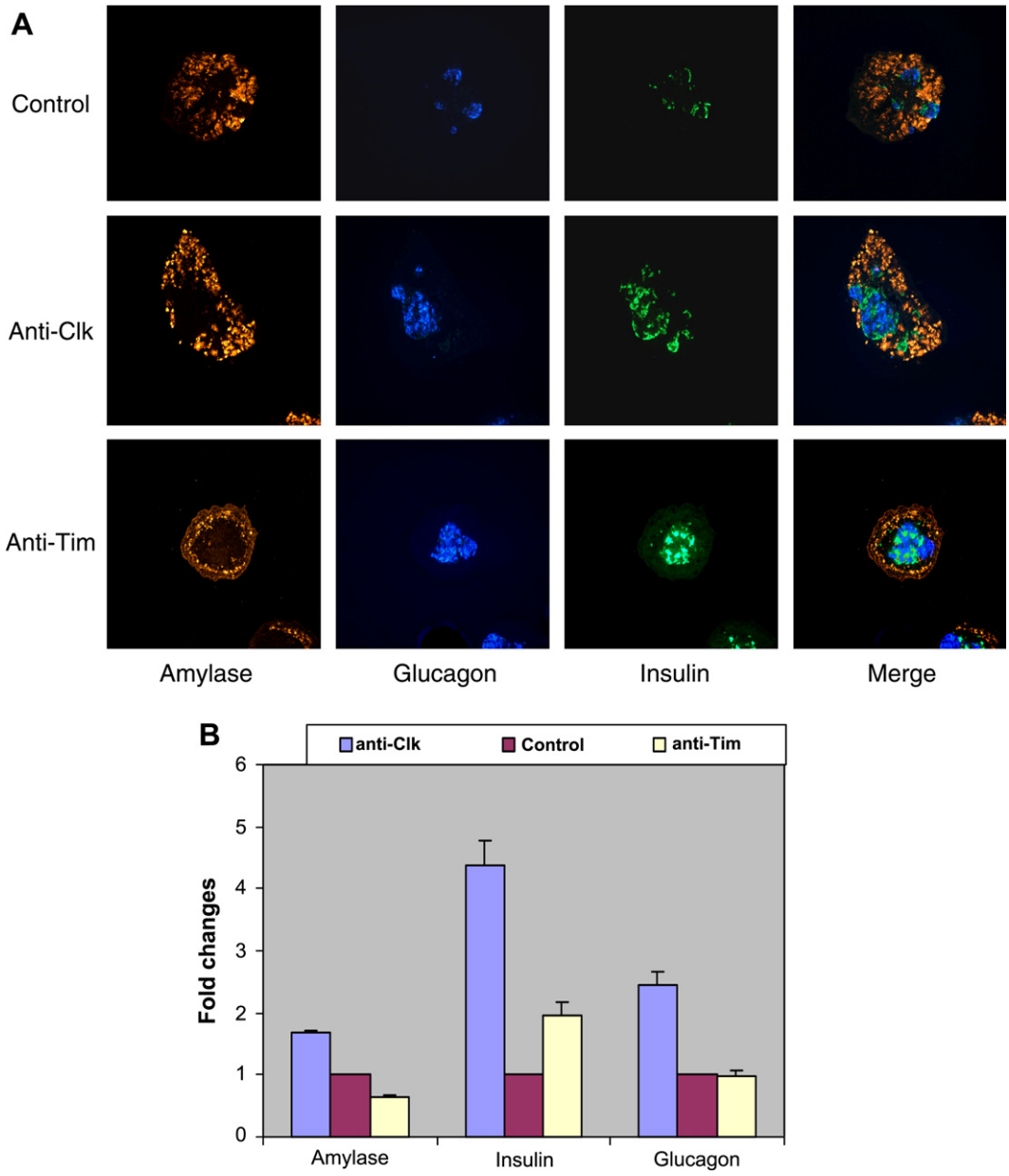

Fig. 3. Circadian gene antisense inhibition study. Early E11 pancreas was isolated and cultured in triplicate in 3-D collagen gel for 6 days with 40 μM of scramble or antisense oligos ($n = 5$). Upper panel: representative staining for each conditions; lower panel: statistical analysis of the positive areas under each condition as compared to control conditions, which were designated as 1. Note the altered selection of endocrine (insulin and glucagons) or exocrine (amylase) lineages.

nucleocytoplasmic shuttling activity of Clock (and Timeless) is dependent on pancreatic differentiation stage (Fig. 2), our data indicate that circadian elements may have a role in the timing of tissue differentiation during embryonic development.

Inhibition of Clock or Timeless displaces Wnt- and Notch-signaling as well as cell cycle components with little effects on pancreatic transcription factors

Some Notch- and Wnt-signaling components have been shown to oscillate and serve as the timing device during somitogenesis [21–23]. Meanwhile, it has been revealed that some cell cycle elements are regulated by circadian genes [24]. Furthermore, evidence from gene chip and microarray analysis has uncovered that circadian gene oscillations, especially Clock oscillations, are the direct or indirect source of all other numerous molecular oscillations [25,26]. Thus, we hypothesize that circadian genes such as Clock and Timeless may serve as the timing device for embryonic tissue differentiation by employing similar Notch- and Wnt-based components in somitogenesis and those elements in cell cycle regulation.

Shown in Fig. 4, RT-PCR analysis revealed that inhibition of circadian genes (Clock/Tim) disturbed the expression of most of the Notch- and Wnt-signaling

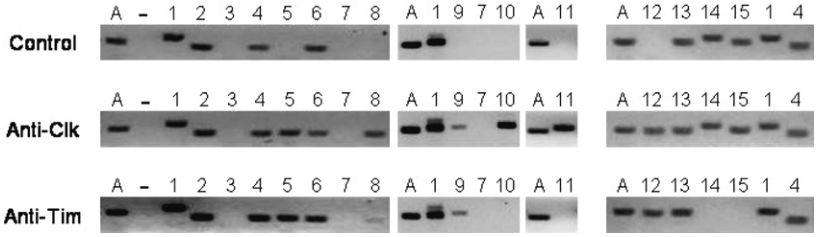

Fig. 4. RT-PCR analysis of Notch- and Wnt-signaling components, cell cycle elements and pancreatic transcription factors. (A) β-actin; −, negative control; 1, Ngn3; 2, Pdx1; 3, HNF3β; 4, Hes1; 5, Notch1; 6, Notch3; 7, Hes7; 8, Delta1; 9, Lunatic Fringe; 10, Notch2; 11, Jag1; 12, Cyclin B1; 13, P34(cdc2); 14, Wee1; 15, Axin2.

components as well as cell cycle elements regulated in the timing of tissue differentiation, i.e., expression of the implicated timing molecules was either affected by Clock (Notch1 and 2, Delta1, Cyclin B1, Lunatic Fringe, and JAG1) or Timeless (Delta1, Cyclin B1, Lunatic Fringe, Wee1, and Axin2) antisense treatment. On the other hand, the circadian gene inhibition had little influence on the expression of pancreatic transcription factors [27] such as Pdx1, Ngn3, and Hes1. Thus, in the pancreas differentiation, circadian genes (Clock, Timeless, etc) might provide the original oscillation, which modulates cell cycle, Wnt- and Notch-signaling components, determining the timing of tissue differentiation.

Discussion

In the circadian mechanism, daily cycle is activated by the expression of Clock, which forms heterodimer with BMAL1. The Clock/BMAL1 complex then translocates into the nucleus and binds on the e-box on Pers, Crys, Rev-Erb and Dels and Tim to drive their expressions. The expression of these circadian elements in return, forms negative feedback loops respectively to suppress Clock activations, thus closing the 24-h cycle [19]. Therefore, in this situation, the nucleocytoplasmic shuttling of Clock correlates with the external day/night cue.

Since embryonic circadian rhythms only appear shortly before birth, the expression of core circadian genes such as Timeless and Clock during early embryonic development before circadian rhythms happen might suggest their potential functions in the embryo development. We have previous found that the core circadian gene Timeless took part in epithelial morphogenesis during kidney development, first revealed the circadian gene's role in the process of mammalian embryonic development. Here we report the detection of Clock expression during mouse embryogenesis and the nucleocytoplasmic shuttling of Clock in the early post implantation embryo and the developing pancreas. Most importantly, this nucleocytoplasmic shuttling of Clock is not coupled with the day/night cue but correlated with stages of cell/tissue differentiation, suggesting its related function in differentiation.

Inhibition of both Clock and Timeless did not block the process of pancreatic differentiation but induce changes in the balance and maturity of endocrine/exocrine cells. So the nucleocytoplasmic shuttling of Clock in the E13–E15 pancreas (Fig. 2) may function in the timing of pancreas differentiation. The changes (area and intensity) in endocrine and exocrine expression could be attributed to the alteration of pace (timing) for differentiation, which is regulated by circadian elements such as Clock and Timeless. RT-PCR data show that inhibition of both Clock and Timeless disturb the expression of Notch- and Wnt-signaling components and some cell cycle elements, all of which have been reported to function in timing regulation. Thus, it is possible that in the process of pancreatic differentiation, the oscillation of circadian gene expression acts as a master controller of other potential timing oscillators such as Notch- and Wnt-signaling loops, and cell cycle regulation, and serves as the ultimate governor for the clock of embryonic differentiation.

In conclusion, the detection of expression and nucleocytoplasmic shuttling of Clock gene in early embryonic stages when the embryonic circadian rhythms have not established suggests Clock's function in the timing regulation of embryo development. Our observation first implies Clock's diverse biological activities and adds to the weight for its non-circadian function during embryogenesis.

Acknowledgments

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