Ultradian Oscillators in Somite Segmentation and Other Biological Events

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Somite formation occurs every 2 hours in mouse embryos by periodic segmentation of the anterior ends of the presomitic mesoderm, and this process is controlled by a biological clock called the segmentation clock. During this process, the basic helix-loop-helix gene *Hes7* is cyclically expressed, and each cycle leads to generation of a bilateral pair of somites. Both sustained expression and loss of expression of *Hes7* result in severe somite fusion, indicating that *Hes7* constitutes an essential component of the segmentation clock. Interestingly, expression of the related gene *Hes1* also oscillates with a periodicity of about 2 hours in many cell types. Both sustained *Hes1* expression and loss of *Hes1* activity lead to retardation of the G₁ phase of the cell cycle, suggesting that *Hes1* oscillation with an ultradian rhythm is required for efficient cell proliferation. Both *Hes1* and *Hes7* oscillations are regulated by negative feedback and rapid degradation of their gene products. Strikingly, expression of other factors such as Stat-Socs and Smad signaling molecules also display ultradian rhythms. All of these data suggest that ultradian oscillations are more general responses than were previously thought and that oscillatory and sustained gene expression results in different biological outcomes.

INTRODUCTION

It is well known that many day and night activities have a circadian rhythm, which has a periodicity of about 24 hours. Some biological activities, however, are known to have rhythms with shorter periodicities called ultradian rhythms. For example, secretion of hormones and changes of plasma glucose levels are pulsatile with periodicities of a few hours (Tannenbaum and Martin 1976; Simon et al. 1987). Another example is somite formation, which occurs every 2 hours in the mouse and every several hours in humans. Recently, characterization of the molecular mechanism of periodic somite formation has been greatly advanced.

Somites are transient metameric structures located at either side of the neural tube, and they later give rise to the vertebral column, ribs, skeletal muscles, and subcutaneous tissues. Somites are formed by segmentation of the anterior ends of the presomitic mesoderm (PSM) (Fig. 1a). In mouse embryos, a bilateral pair of somites are formed every 2 hours, suggesting that this process is controlled by a biological clock with a 2-hour periodicity, and it has been called the segmentation clock (Bessho and Kageyama 2003; Pourquié 2003; Aulehla and Herrmann 2004; Gridley 2006). During this process, the basic helix-loop-helix (bHLH) genes *Hes1* and *Hes7* display oscillatory expression in PSM cells, leading to generation of a pair of somites after each cycle (Bessho et al. 2001a). In particular, *Hes7* is an essential component of the segmentation clock. In the absence of *Hes7*, somites become severely fused (Bessho et al. 2001b). Interestingly, *Hes1* also shows oscillatory expression in many cell types, in addition to PSM cells, suggesting that oscillation is not unique to PSM cells and that *Hes1* oscillation may regulate the timing of many biological events (Jouve et al. 2000; Hirata et al. 2002). It has been shown that other

genes are also cyclically expressed by many cell types, raising the possibility that oscillatory expression is more general than was previously thought. In this chapter, we

Figure 1. *Hes7* oscillation in the somite segmentation. (a) In mouse embryos, a bilateral pair of somites are formed every 2 hours by segmentation of the anterior ends of the PSM (asterisk). Expression of the *Hes7* intron (corresponding to the region where the *Hes7* gene is transcribed, green) is initiated in the posterior PSM (phase I), and this expression region is then propagated into the anterior PSM (phase II), finally reaching S-1 (phase III). By the time S-1 becomes S0, *Hes7* intron expression disappears, and the next expression occurs again in the posterior PSM (i.e., it returns to phase I). The *Hes7* transcription region (green) and the *Hes7*-protein-expressing region (blue) are mutually exclusive in all three phases, suggesting that *Hes7* transcription occurs only when *Hes7* protein is not expressed. S0 is the next somitic region formed, whereas S-1 is the region that becomes a somite following S0. Anterior is top. (b) Dynamic change in *Hes7* expression is the result of oscillatory expression in individual PSM cells (indicated by a dot in a). (Adapted, with permission, from Kageyama et al. 2007a [© John Wiley & Sons].)

discuss the mechanism and the significance of *Hes1*/*Hes7* oscillations and other ultradian oscillators in biological events.

THE ROLES AND THE MECHANISM OF *HES7* OSCILLATION IN THE SEGMENTATION CLOCK

Hes7 expression dynamically changes in PSM and has been classified as occurring in three phases (Fig. 1a). Expression of the *Hes7* intron (corresponding to the region where *Hes7* gene is transcribed) is initiated in the posterior PSM (phase I), and this region is then propagated into the anterior PSM (phase II), finally reaching S-1 (phase III) (Fig. 1a, green). By the time S-1 becomes S0 (the next somitic region formed), *Hes7* intron expression disappears, whereas the next expression of *Hes7* intron occurs again in the posterior PSM (i.e., it returns to phase I). This dynamic change in gene expression is the result of oscillatory expression of *Hes7* in individual PSM cells (Fig. 1b). Cells located laterally display oscillation in-phase, but this is delayed in anterior cells compared to posterior cells. As a result, the expression domain moves from the posterior to the anterior region.

Interestingly, the *Hes7* transcription region (Fig. 1a, green) and the *Hes7* protein-expressing region (Fig. 1a, blue) are mutually exclusive in all three phases, suggesting that *Hes7* transcription occurs only when *Hes7* protein is not expressed (Bessho et al. 2001a, 2003). In agreement with this notion, the *Hes7* protein can repress *Hes7* transcription by directly binding to its own promoter (Bessho et al. 2003). Furthermore, in the absence of functional *Hes7* protein, the *Hes7* gene is constitutively transcribed in the PSM, whereas *Hes7* transcription is constitutively repressed when *Hes7* protein is stabilized (Bessho et al. 2003). These data together suggest that *Hes7* oscillation is regulated by negative autoregulation as follows (Fig. 2). In the PSM, Notch signaling induces expression of *Hes7*, which represses its own expression by binding to its own promoter. When the promoter is repressed, both *Hes7* mRNA and protein disappear rapidly because they are extremely unstable, allowing the next round of expression. In this way, *Hes7* autonomously starts oscillatory expression.

Figure 2. *Hes7* oscillation regulated by negative autoregulation. Notch signaling induces expression of *Hes7*, which represses its own expression by binding to its own promoter. When the promoter is repressed, both *Hes7* mRNA and protein disappear rapidly because they are extremely unstable, allowing the next round of expression. In this way, *Hes7* autonomously starts oscillatory expression.

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Hes1 displays an expression pattern similar to that of *Hes7* in the PSM. *Hes1*, a transcriptional repressor, can repress its own expression by directly binding to the promoter (Sasai et al. 1992; Takebayashi et al. 1994), and *Hes1* oscillation is also regulated by this negative autoregulation (Hirata et al. 2002), although the expression is maintained in S0 and in the somitic regions, unlike *Hes7*.

It has been shown that expression of *Lunatic fringe* (*Lfng*), a glycosyltransferase gene that inhibits Notch activity (Brückner et al. 2000; Moloney et al. 2000), oscillates in phase with *Hes7* (Bessho et al. 2001b). In *Lfng*-null mice, *Hes7* expression still oscillates, although the expression is somewhat affected (Niwa et al. 2007), suggesting that *Hes7* oscillation does not depend on *Lfng* oscillation. In contrast, in *Hes7*-null mice, *Lfng* is constitutively expressed throughout the PSM (Bessho et al. 2001b). Conversely, stabilized *Hes7* constitutively represses *Lfng* expression (Bessho et al. 2003). These data indicate that *Lfng* oscillation depends on periodic repression by *Hes7*.

Both *Hes7* and *Lfng* have an essential role in somite segmentation. In *Hes7*-null and *Lfng*-null mice, somites severely fuse (Evrard et al. 1998; Zhang and Gridley 1998; Bessho et al. 2001b). Interestingly, transgenic mice that constitutively express *Hes7* or *Lfng* throughout the PSM also display severe somite fusion (Serth et al. 2003; Hirata et al. 2004). Thus, both sustained expression and loss of expression of *Hes7* or *Lfng* disturb somite segmentation, suggesting that oscillatory expression of both genes is required for this process.

MATHEMATICAL SIMULATION OF *HES7* OSCILLATION

Hes1/*Hes7* oscillations can be mathematically simulated, based on the negative autoregulation mechanism described above (Jensen et al. 2003; Lewis 2003; Monk 2003; Hirata et al. 2004). The simulation for *Hes7* oscillation consists of two differential equations (Hirata et al. 2004).

$$\frac{dp(t)}{dt} = am(t - T_p) - bp(t)$$

$$\frac{dm(t)}{dt} = f(p(t - T_m)) - cm(t)$$

$$f(p) = \frac{k}{1 + \left(\frac{p}{p_{crit}}\right)^2}$$

where $p(t)$ and $m(t)$ are the quantities of functional *Hes7* protein and mRNA, respectively, per cell at time t , and $f(p)$ is the rate of initiation of transcription, which is negatively regulated by a dimer of the protein p present at the time of initiation. a is the rate constant for translation, whereas b and c are the degradation rate constants for protein and mRNA, respectively.

According to this simulation, the instability of *Hes7* protein is very important for sustained oscillation. The

Figure 3. Stabilization of Hes7 protein leads to damped oscillation. The half-life of Hes7 protein is about 20 minutes, and this value is important for sustained oscillations. If the half-life becomes 30 minutes, Hes7 oscillations become damped after three to four cycles. (Adapted, with permission, from Hirata et al. 2004 [Nature Publishing Group].)

half-life of Hes7 protein is about 20 minutes, but if it reaches 30 minutes instead of 20 minutes, the simulation predicts that Hes7 oscillation becomes damped after several cycles (Fig. 3). However, this prediction is counterintuitive, which suggests that even if the half-life is 30 minutes, Hes7 oscillations could continue in a sustained manner, although the period would be a little longer than 2 hours. The prediction of the mathematical simulation was experimentally evaluated by making mutant mice that express stabilized Hes7 protein. Hes7 protein with a K14R mutation (the 14th amino acid residue [lysine] of Hes7 protein is mutated to arginine) has a normal repressor activity, but its half-life is about 30 minutes. A K14R point mutation was introduced into the *Hes7* locus of ES cells, and from these ES cells, the mutant mice that express Hes7 with the K14R mutation were generated. Interestingly, although three or four pairs of somites are segmented, the subsequent somites fuse severely in these mutant mice (Hirata et al. 2004). Furthermore, Hes7 oscillation becomes damped after three to four cycles (Hirata et al. 2004). These data agree well with the prediction of the mathematical simulation and indicate that intuition alone is insufficient and that mathematical modeling is required to understand the dynamics of Hes oscillations.

REAL-TIME MONITORING OF *Hes* OSCILLATIONS

To further examine Hes oscillation patterns, we established a real-time imaging system for Hes1/Hes7 expression, where the Hes1/Hes7 promoter-driven ubiquitinated luciferase is used as a reporter (Masamizu et al. 2006; and our unpublished data). Because the normal luciferase protein has a half-life of several hours, it is too stable to monitor 2-hour cycle oscillations. Such a reporter protein would be accumulated after several cycles of oscillations. In contrast, the ubiquitinated luciferase has a half-life of about 10 minutes and can be used for monitoring oscillations with a 2-hour periodicity. The system with the ubiquitinated luciferase reporter successfully enabled observation of Hes1/Hes7 oscillations in real time in PSM, offering a powerful tool to understand the dynamics of Hes1/Hes7 oscillations. On

the basis of real-time imaging, a spatiotemporal profile was reproduced (Fig. 4a) (Kageyama et al. 2007a). This profile suggests that each individual PSM cell seems to experience five cycles of Hes1 oscillations before entering the S0 region (the next somite region) about embryonic day 10.5. Interestingly, the period of the first three cycles is about 2 hours, whereas that of the fourth and fifth cycles is longer than 2 hours (Fig. 4b). Cells experiencing the first three cycles are located in the posterior PSM, whereas those experiencing the last two cycles are located in the anterior PSM, indicating that the period of Hes1 oscillations is different between the posterior and the anterior PSM. The different periods between the posterior and the anterior PSM could be regulated by Fgf signaling (Niwa et al. 2007), which forms a posterior-to-anterior gradient in the PSM, although the precise mechanism remains to be determined.

Hes1 expression oscillates in a stable manner in PSM cells but not in dissociated PSM cells. In these cells, the periods of Hes1 oscillations are variable from cycle to cycle, indicating that Hes1 oscillation in individual PSM cells is unstable (Masamizu et al. 2006). Thus, negative autoregulation can create only an unstable oscillator, suggesting that cell–cell communication and/or extracellular signals are important for stable Hes1 oscillations. We observed the same features in Hes7 oscillations (our unpublished data). It is likely that Notch signaling is involved in cell–cell communication (Jiang et al. 2000; Horikawa et al. 2006), although further analysis is required to clarify this issue.

Figure 4. Spatiotemporal profile of Hes1 oscillation in PSM. (a) A spatiotemporal profile of Hes1 oscillation in PSM was reproduced, based on the real-time imaging analysis (Masamizu et al. 2006; Kageyama et al. 2007a). (b) Hes1 expression in individual PSM cells. Each PSM cell seems to experience five cycles of Hes1 oscillation after getting out of the tail bud and before reaching the S0 region. The periodicity of the first three cycles is about 2 hours, whereas that of the last two cycles is longer, suggesting that Hes1 oscillation is differentially regulated between the anterior and the posterior PSM. (Adapted, with permission, from Kageyama et al. 2007a [© John Wiley & Sons].)

HES1 OSCILLATION IN NON-PSM CELLS

Interestingly, oscillatory expression is not unique to PSM cells. Hes1 is widely expressed by many cell types such as fibroblasts and neuroblasts (Kageyama et al. 2007b). After serum stimulation or Notch activation, Hes1 expression oscillates with a periodicity of about 2 hours in many cell types, suggesting that Hes1 may regulate many biological processes as a biological clock with a 2-hour cycle (Hirata et al. 2002). Both sustained Hes1 expression and knock down of Hes1 activity reduce proliferation of fibroblasts, suggesting that oscillatory Hes1 expression is required for efficient proliferation of fibroblasts (Yoshiura et al. 2007). Furthermore, persistent Hes1 expression also inhibits proliferation and differentiation of neuroblasts (Baek et al. 2006). It seems that Hes1 promotes certain steps but inhibits other steps of the cell cycle, although it remains to be determined at which steps of the cell cycle Hes1 regulation actually occurs.

Northern and western blot analyses indicate that in non-PSM cells, Hes1 oscillations seem to be damped after three to six cycles (Hirata et al. 2002). Real-time monitoring at the single-cell level revealed that Hes1 oscillation continues in each individual cell even after 2 days, but because the period of each cycle is variable from cycle to cycle and from cell to cell, Hes1 oscillation among cells soon goes out of synchrony (Masamizu et al. 2006). Thus, damping of Hes1 oscillation in the population is not due to damped oscillation in all individual cells, but it is due to desynchronization between cycling cells. Interestingly, this unstable period of Hes1 oscillation of fibroblasts is very similar to that of dissociated PSM cells.

OTHER ULTRADIAN OSCILLATORS

In addition to Hes1/Hes7, many other factors have been shown to display oscillatory expression in PSM cells (Dequéant et al. 2006). Similarly, several other factors have been shown to display oscillatory expression in non-PSM cells. NF- κ B is trapped in the cytoplasm by I κ B, but in the presence of inducing signals such as tumor necrosis factor- α (TNF- α), I κ B is degraded, and NF- κ B is released and transferred into the nucleus, where it induces expression of many genes. One of them is I κ B, which again traps NF- κ B. However, if the inducing signals are still present, I κ B is degraded and NF- κ B is translocated to the nucleus again. As a result, the localization of NF- κ B oscillates between the nucleus and cytoplasm (N-C oscillation) (Hoffmann et al. 2002; Nelson et al. 2004). It has been shown that p53 expression also oscillates due to the negative feedback of Mdm2 (Bar-Or et al. 2000; Lahav et al. 2004). These observations suggest the possibility that other molecules forming negative feedback loops can also display oscillatory responses.

To identify new ultradian oscillators, RNA was prepared from fibroblasts every 30 minutes after serum stimulation and subjected to microarray analyses, which revealed oscillatory responses of Socs3 and Smad6 (Yoshiura et al. 2007). Expression of both genes oscillates with a period of about 2 hours, although the peaks appear at different times. Socs3 is the downstream target of the

Jak-Stat signaling (Starr et al. 1997; Levy and Darnell 2002; Yu and Jove 2004). Jak2 activates Stat3 by phosphorylation, and phosphorylated Stat3 (p-Stat3) forms a dimer, enters the nucleus, and induces expression of many genes (Fig. 5). One of them is Socs3, which antagonizes Jak-dependent activation of Stat3, thus forming a negative feedback loop. Because Socs3 expression oscillates, Jak-dependent activation of Stat3 should be periodically inhibited. In agreement with this notion, formation of p-Stat3 is also found to be oscillatory. Thus, the Jak-Stat3-Socs3 negative feedback loop generates p-Stat3/Socs3 oscillations (Fig. 5) (Yoshiura et al. 2007). Importantly, inhibition of p-Stat3/Socs3 oscillations abolishes Hes1 oscillation, suggesting that Hes1 oscillation is not solely regulated by negative feedback. p-Stat3 seems to periodically destabilize Hes1 protein, and this periodic destabilization may be important for Hes1 oscillation (Yoshiura et al. 2007). Conversely, it was previously shown that Hes1 promotes Jak-dependent activation of Stat3 by physical interaction (Kamakura et al. 2004). Thus, it is likely that Hes1 oscillation and p-Stat3/Socs3 oscillations are coupled with and depend on each other.

Smad6 is the downstream target of Smad1 signaling (Imamura et al. 1997; Massagué and Wotton 2000; ten Dijke et al. 2000). Signaling molecules such as bone morphogenetic protein (BMP) activate Smad1/5/8 by phosphorylation. Phosphorylated Smad1/5/8 (p-Smad1/5/8) up-regulates expression of downstream genes including Smad6, which then inhibits p-Smad1/5/8 formation, constituting a negative feedback loop (Fig. 6). Because Smad6 expression oscillates, Smad1/5/8 activation should be periodically inhibited. In agreement with this notion, formation of p-Smad1 is also found to be oscillatory. Thus, the Smad1-Smad6 negative feedback loop generates p-Smad1/Smad6 oscillations (Fig. 6) (Yoshiura et al. 2007). However, unlike p-Stat3/Socs3 oscillations, p-Smad1/Smad6 oscillations are not involved in Hes1 oscillation, suggesting that the p-Smad1/Smad6 oscillations function independently of Hes1 oscillation. In

Figure 5. p-Stat3/Socs3 oscillations. Jak2 activates Stat3 by phosphorylation. Phosphorylated Stat3 (p-Stat3) enters the nucleus and induces expression of downstream genes. One of them is Socs3, which antagonizes formation of p-Stat3. This negative feedback loop generates oscillatory expression of p-Stat3 and Socs3.

Figure 6. p-Smad1/Smad6 oscillations. Signaling molecules such as BMP activate Smad1/5/8 by phosphorylation. Phosphorylated Smad1/5/8 (p-Smad1/5/8) up-regulates expression of downstream genes including Smad6, which then inhibits p-Smad1/5/8 formation, constituting a negative feedback loop. Because Smad6 expression oscillates, Smad1/5/8 activation should be periodically inhibited. In agreement with this notion, formation of p-Smad1 is found to be oscillatory. The Smad1/5/8-Smad6 negative feedback loop generates p-Smad1/5/8 and Smad6 oscillations.

agreement with this notion, Hes1 and Socs3 oscillate in phase, whereas Hes1 and Smad6 do not.

POSSIBLE SIGNIFICANCE OF ULTRADIAN OSCILLATIONS

Although Hes1 oscillation is required for efficient cell proliferation, the expression modes of the downstream target genes are not well-characterized. In our microarray analyses, we did not identify oscillatory downstream target genes for Stat, Smad, and Hes oscillators. Expression of some downstream genes could oscillate (Fig. 7a), but synchrony of oscillation among cells might easily be lost. In this case, real-time monitoring of gene expression at the single-cell level is required. Hes1 oscillations would produce different conditions in different cells, leading to different responses. For example, when differentiation is induced, cells in a low Hes1 phase, but not those in a high Hes1 phase, would respond to the induction. Thus, Hes1 oscillations could make responsive and unresponsive cells to the same signals.

If the downstream target gene products are relatively stable, they do not respond in an oscillatory manner. For example, expression of such downstream factors can be maintained within a certain range by periodic down-regulation by Hes1 oscillation (Fig. 7b). If products of downstream target genes are even more stable, transcriptional induction by activators and periodic repression by Hes1 oscillation could lead to up-regulation of downstream target factors in a stepwise manner (Fig. 7c). In this case, if the expression level reaches a certain value, the next event could happen. Thus, the information about the number of cycles can be converted into the timing of the next event. Further analysis of downstream target genes will be required to reveal more precise significance and roles of ultradian oscillators.

Figure 7. Downstream events of ultradian oscillators. Downstream factors for Hes1/Hes7 oscillations could display at least three different expression patterns. (a) If products of downstream genes are unstable, their expression could oscillate like Hes1 and Hes7. (b) If downstream factors are relatively stable, their expression can be maintained within a certain range by periodic down-regulation by Hes1/Hes7 oscillations. (c) If downstream factors are very stable, transcriptional induction by activators and periodic repression by Hes1/Hes7 oscillations could lead to up-regulation of downstream target factors in a stepwise manner. If the expression level reaches a certain value, the next event could happen. In this case, the information about the number of cycles can be converted into the timing of the next event.

CONCLUSIONS

It has been shown that many genes display expression with ultradian rhythms. In the case of Hes1, although the expression seems to be stationary on northern and western analyses without serum stimulation, it is actually oscillatory at the single-cell level. Oscillatory expression of Hes1 is required for efficient cell proliferation, as sustained expression retards the G₁ phase of the cell cycle. Similarly, oscillatory expression of Hes7 is required for somite segmentation, as sustained expression leads to severe somite fusion. Thus, oscillatory and sustained gene expression result in different biological outcomes. Ultradian oscillations are regulated by negative feedback, but it seems that the negative feedback loop alone generates only unstable oscillators. Cell-cell communication or other extracellular signals are required for stable oscillators. Reconstitution of such oscillators in non-PSM cells

would be required to understand the basic mechanism of stable oscillations.

Other molecules forming negative feedback loops are also shown to display ultradian rhythms, suggesting that ultradian oscillations are more general responses than were previously thought. Further characterization of ultradian oscillators and their downstream targets will give us more insight into the understanding of how cells measure time in many biological processes.

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