Mol. Cells *27*, 497-502, May 31, 2009
DOI/10.1007/s10059-009-0068-1

Minireview

Molecules and Cells

Rhythmic Gene Expression in Somite Formation and Neural Development

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In mouse embryos, somite formation occurs every two hours, and this periodic event is regulated by a biological clock called the segmentation clock, which involves cyclic expression of the basic helix-loop-helix gene *Hes7*. Hes7 expression oscillates by negative feedback and is cooperatively regulated by Fgf and Notch signaling. Both loss of expression and sustained expression of Hes7 result in severe somite fusion, suggesting that Hes7 oscillation is required for proper somite segmentation. Expression of a related gene, *Hes1*, also oscillates by negative feedback with a period of about two hours in many cell types such as neural progenitor cells. *Hes1* is required for maintenance of neural progenitor cells, but persistent *Hes1* expression inhibits proliferation and differentiation of these cells, suggesting that *Hes1* oscillation is required for their proper activities. Hes1 oscillation regulates cyclic expression of the proneural gene *Neurogenin2* (*Ngn2*) and the Notch ligand *Delta1*, which in turn lead to maintenance of neural progenitor cells by mutual activation of Notch signaling. Taken together, these results suggest that oscillatory expression with short periods (ultradian oscillation) plays an important role in many biological events.

INTRODUCTION

During embryogenesis, many events occur coordinately according to the scheduled time course. For example, in mouse embryos, gastrulation begins around embryonic day (E) 6.5, the neural plate is formed around E7.5, the forelimb buds appear around E9.5, and so on, and finally gestation ends in 19-20 days after fertilization. During these processes, thousands of genes are expressed coordinately in good time, suggesting that the timing of gene expression is controlled by some biological clocks. However, such clocks remain largely unknown, with one exception: the somite segmentation clock, which regulates periodic somite formation (Aulehla and Herrmann, 2004; Dequéant and Pourquié, 2008; Gridley, 2006; Kageyama et al., 2007). In mouse embryos, a pair of somites is generated every two hours. During this process, many genes are rhythmically expressed (two hour periodicity), and this rhythmic expression is essential for periodic somite formation. Recent studies revealed that oscillatory gene expression with a period of about two to several hours (ultradian rhythms) is not unique to the cells forming somites but is observed in many cell types such as neural progenitor cells (Hirata et al., 2002; Pascoal et al., 2007; Shimojo et al., 2008). In this review, we describe recent findings about rhythmic gene expression in somite formation and neural development and discuss that not only gene expression but also the expression mode (rhythmic versus sustained) is important for many biological events.

Dynamic Hes7 expression regulates somite segmentation

Somites, transient metameric structures, are precursors for vertebrae, ribs, skeletal muscles and subcutaneous tissues. Somites are formed by periodic segmentation of the anterior ends of the presomitic mesoderm (PSM), which is located in the posterior part of embryos. This periodic event is regulated by the segmentation clock whose periodicity is species-specific: zebrafish, 30 min; chick, 90 min; mice, 2 h; and human 6-8 h. It has been shown that the basic helix-loop-helix (bHLH) gene *Hes7* plays an essential role in the segmentation clock in mouse embryos (Bessho et al., 2001). *Hes7* expression (transcription) starts in the posterior PSM (phase I), is propagated anteriorly (phase II), and stops near the anterior ends of the PSM (phase III) (Fig. 1A). Then, the expression in the anterior PSM disappears and segmentation follows, forming a bilateral pair of somites, while new expression starts in the posterior PSM (returns to phase I) (Fig. 1A). In *Hes7*-null mice, the somites are severely fused, indicating that *Hes7* is required for somite segmentation (Bessho et al., 2001). Interestingly, mice expressing Hes7 constitutively in the PSM also display severe somite fusion (Niwa et al., 2007). Thus, both loss of expression and sustained expression of Hes7 result in severe somite fusion, suggesting that Hes7 oscillation is required for periodic somite segmentation.

Mechanism for Hes7 oscillation and mathematical modeling

The dynamic expression of *Hes7*, which is repeated every two hours in the mouse PSM, does not depend on cell movement

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Received March 17, 2009; accepted March 20, 2009; published online May 15, 2009

Keywords: biological clock, negative feedback, segmentation clock, ultradian oscillation

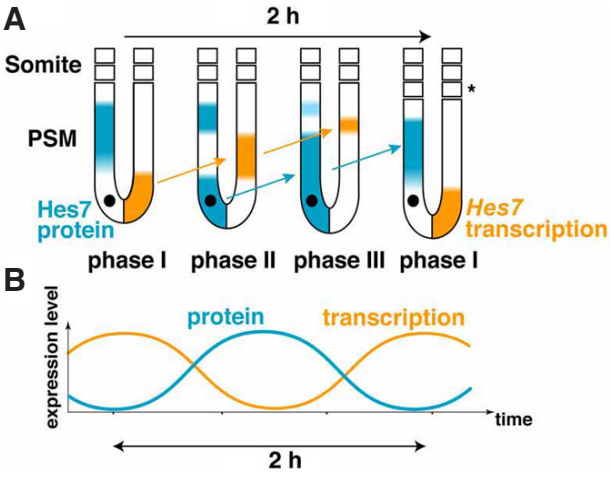

Fig. 1. Hes7 oscillation in the PSM. (A) In mouse embryos, a bilateral pair of somites is formed every two hours by segmentation of the anterior ends of the PSM (asterisk). Expression of the *Hes7* intron (corresponding to *Hes7* gene transcription) is initiated in the posterior PSM (phase I), and this expression region moved into the anterior PSM (phase II), and finally stops near the anterior ends (phase III). Then, segmentation occurs and *Hes7* intron expression in the anterior PSM disappears, while the next expression occurs again in the posterior PSM (i.e., it returns to phase I). The *Hes7* gene transcription and the Hes7 protein expression are mutually exclusive in all three phases, suggesting that *Hes7* transcription occurs only when Hes7 protein is not expressed. 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)).

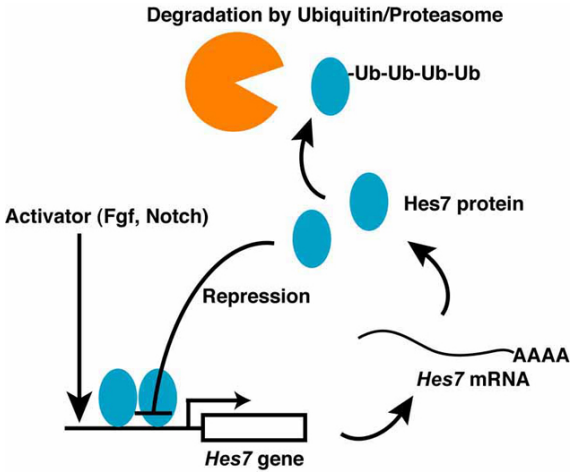

Fig. 2. Regulation of Hes7 oscillations by negative feedback and rapid degradation of gene products. Activation of *Hes7* promoter leads to synthesis of *Hes7* mRNA and Hes7 protein. Hes7 protein then represses its own expression by directly binding to the *Hes7* promoter, and thus transcription of the Hes7 locus does not occur when Hes7 protein is expressed. When the transcription is repressed by such negative feedback, both *Hes7* mRNA and Hes7 protein disappear rapidly because they are extremely unstable, allowing the next round of expression. In this way, *Hes7* autonomously starts rhythmic expression.

but is the result of oscillatory expression in individual PSM cells (Fig. 1B). This oscillatory expression is regulated by negative feedback and the instability of the gene products (Bessho et al., 2003; Hirata et al., 2004). Hes7 protein can repress its own expression by directly binding to the *Hes7* promoter (Fig. 2), and thus transcription of the Hes7 locus does not occur when Hes7 protein is expressed (Figs. 1A and 1B) (Bessho et al., 2003; Chen et al., 2005). When the transcription is repressed by such negative feedback, both Hes7 mRNA and protein disappear rapidly because they are extremely unstable, allowing the next round of expression (Fig. 2) (Bessho et al., 2003). In this way, *Hes7* autonomously starts rhythmic expression.

Based on the negative feedback mechanism, oscillatory expression such as Hes7 oscillation has been mathematically simulated with differential equations (Hirata et al., 2004; Jensen et al., 2003; Lewis, 2003; Monk, 2003):

$$dp(t)/dt = am(t - T_p) - bp(t)$$
$$dm(t)/dt = k/[1 + \{p(t - T_m)\}^2/p_0{}^2] - cm(t)$$

where $p(t)$ and $m(t)$ are the quantities of functional Hes7 protein and *Hes7* mRNA per cell at time t, respectively, and p_0 is the amount of protein that shows half-maximal inhibition. a is the rate constant for translation, while b and c are the degradation rate constants for Hes7 protein and *Hes7* mRNA, respectively. These equations successfully simulated two-hour cycle oscillation of Hes7. This mathematical model predicts that the degradation rate constants should be large (the gene products should be unstable) to sustain oscillatory expression, and if these constants are small, the oscillation would be damped. To evaluate this prediction, we made mice expressing stabilized Hes7 protein (reduced degradation rate) by introducing a point mutation into the Hes7 locus. In these mice, Hes7 oscillation was damped after three or four cycles of oscillation, resulting in somite fusion (Hirata et al., 2004). These results indicate that sustained Hes7 oscillation depends on the instability of gene products.

Coupled oscillations underlie the segmentation clock

The initiation of Hes7 expression in the posterior PSM is regulated by Fgf8 (Niwa et al., 2007), an extracellular signaling molecule forming the posterior-anterior gradient (Fig. 3) (Dubrulle and Pourquié, 2004). Hes7 expression is then amplified and propagated into the anterior PSM by Notch signaling, which is activated by Delta from neighboring cells (Fig. 3) (Niwa et al., 2007). In the absence of Fgf signaling, Hes7 expression is lost, whereas in the absence of Notch signaling, Hes7 expression still oscillates in the posterior PSM, although the expression level is very low. Thus, while Hes7 oscillation is regulated by negative feedback, it is also cooperatively regulated by Fgf8 and Notch signaling, intercellular molecules. Conversely, Hes7 oscillation leads to rhythmic expression of Dusp4 and Lunatic fringe (Lfng), inhibitors for Fgf8 and Notch signaling, respectively. As a result, the intracellular activities of both Fgf8 and Notch signaling oscillate coordinately (Fig. 3) (Huppert et al., 2005; Morimoto et al., 2005; Niwa et al., 2007). It is thought that these coupled oscillations involving the intercellular communication are very important for the segmentation clock (Jiang et al., 2000; Horikawa et al., 2006; Riedel-Kruse et al., 2007; Serth et al., 2003). This notion agrees well with the observation that whereas the PSM tissue has robust and stable oscillators, dissociated PSM cells display only very unstable oscillatory expression, suggesting that the intercellular communication is required for stable rhythms (Maroto et al., 2005; Masamizu et al., 2006).

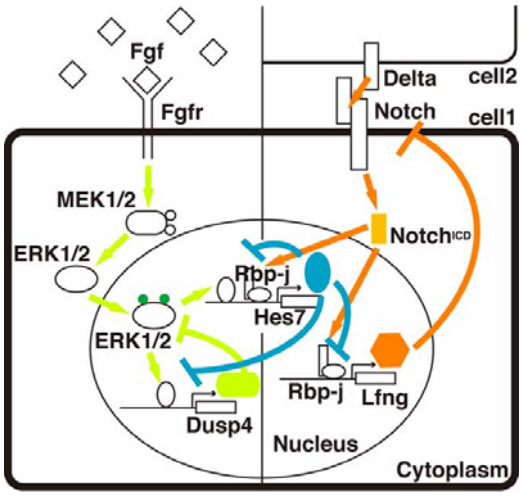

Fig. 3. Hes7-mediated coupled oscillations in Fgf and Notch signaling in the segmentation clock. *Hes7* oscillation is initiated by Fgf signaling and amplified and propagated/maintained cooperatively by Notch signaling. Hes7 oscillations in turn lead to oscillations in phosphorylated ERK (ERK with two green dots) and Notch ICD, Fgf and Notch signaling effectors, respectively. These oscillations coupled by Hes7 seem to play an important role in generation of stable rhythms.

Oscillatory expression is not unique to the PSM cells but was found in many cell types such as neural progenitor cells. In the following sections, we describe the significance of oscillations in neural development.

Roles of bHLH genes in neural development

In the developing nervous system, neural progenitor cells, called neuroepithelial cells, initially undergo self-renewal and thereby proliferate extensively (Fig. 4). As development proceeds, neural progenitor cells are gradually elongated (called radial glial cells) and undergo asymmetric cell division, producing one neural progenitor cell and one neuron/neuronal precursor from each cell division (Fig. 4). After production of neurons, neural progenitor cells differentiate into glial cells such as oligodendrocytes and astrocytes (Fig. 4). Thus, neural progenitor cells change their competency for generation of cell types over time, and thus it is essential to maintain neural progenitor cells until the last stage of development to make the correct numbers and a full diversity of cells.

It has been shown that bHLH genes play a central role in neural development (Bertrand et al., 2002; Kageyama et al., 2005; Ross et al., 2003). There are two types of bHLH genes, proneural/activator-type and repressor-type. Proneural bHLH genes such as *Mash1* and *Neurogenin2* (*Ngn2*) determine the neuronal cell fate, while the repressor-type bHLH genes such as *Hes1* and *Hes5* regulate maintenance of neural progenitor cells by repressing proneural bHLH gene expression (Fig. 4). In the absence of proneural genes, neurons are not formed (Nieto et al., 2001; Tomita et al., 2000), whereas in the absence of repressor-type bHLH genes, proneural gene expression is significantly up-regulated, leading to premature formation of early-born neurons and depletion of neural progenitor cells without generating later-born cell types (Hatakeyama et al., 2004; Ishibashi et al., 1995). Thus, antagonistic regulation between proneural and repressor-type bHLH genes is very important for normal neural development.

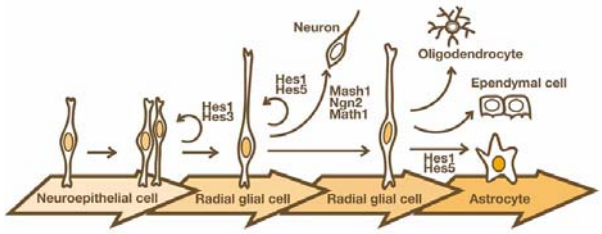

Fig. 4. Neural progenitor cells, neurogenesis and gliogenesis. Neural progenitor cells (neuroepithelial cells) initially proliferate only. As development proceeds, neural progenitor cells are elongated (radial glial cells) and then give rise to neurons by asymmetric cell divisions. After the production of distinct types of neurons, neural progenitor cells finally differentiate into glial cells such as oligodendrocytes and astrocytes. Proneural bHLH genes such as *Mash1* and *Ngn2* determine the neuronal cell fate, while the repressor-type bHLH genes such as *Hes1* and *Hes5* regulate maintenance of neural progenitor cells by repressing proneural bHLH gene expression.

Hes1 and *Hes5* expression are regulated by Notch signaling, which is activated by Notch ligands such as Delta1 (Fig. 5) (Ohtsuka et al., 1999). Proneural bHLH genes induce *Delta1* expression (Castro et al., 2006), and thus differentiating neurons express Notch ligands and activate Notch signaling in neighboring neural progenitor cells (Fig. 5). Upon activation of Notch signaling, the Notch intracellular domain (NICD) is released from the transmembrane region of Notch and transferred to the nucleus, where NICD forms a complex with the DNA-binding protein RBPj (Fig. 5). The complex of NICD-RBPj induces *Hes1* and *Hes5* expression by binding to their promoters (Fig. 5). As a result, proneural genes are repressed, and these cells remain as neural progenitor cells. Thus, neurons inhibit neighboring cells from becoming neurons (lateral inhibition), and it has been suggested that this Notch signaling-mediated lateral inhibition balances formation of neurons with maintenance of neural progenitor cells in the developing nervous system.

However, the Notch signaling-mediated lateral inhibition poses a dilemma. Activation of Notch signaling seems to depend on Notch ligands from neighboring neurons, but how are neural progenitor cells maintained during early stages before neurons are formed? It has been shown that proneural genes and Notch ligands are expressed in salt-and-pepper patterns by neural progenitor cells during early stages (Bettenhausen et al., 1995; Guillemot and Joyner, 1993; Kageyama et al., 2008; Sommer et al., 1996). In the dorsal telencephalon of mouse embryos, neuronal formation starts around E11, but the expression of proneural genes and Notch ligands occur in neural progenitor cells as early as E8.5, suggesting that Notch signaling is mutually activated among neural progenitor cells without any differentiating neurons. However, this raises another question: why cells expressing proneural genes do not differentiate into neurons until E11? The solution of this problem may reside in the expression mode, as stated below.

Oscillatory expression in neural progenitor cells

While *Hes1* is important for maintenance of neural progenitor cells, sustained expression of *Hes1* inhibits proliferation of these cells by G1 phase retardation (Baek et al., 2006). Immunohistochemical studies showed that Hes1 expression levels are not uniform but are variable in neural progenitor cells (Baek et al., 2006). Real-time imaging using the Hes1 promoter-driven

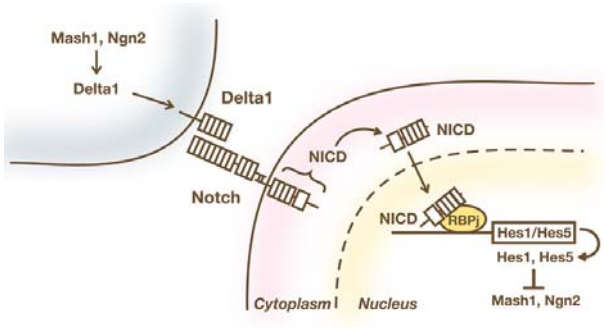

Fig. 5. Notch signaling in the developing nervous system. Proneural genes such as *Mash1* and *Ngn2* induce expression of Notch ligands such as Delta1, which activate Notch signaling of neighboring cells. Upon activation, the Notch intracellular domain (NICD) is released from the transmembrane region and transferred into the nucleus, where NICD forms a complex with RBPj and induces *Hes1* and *Hes5* expression. Hes1 and Hes5 repress proneural gene expression and inhibit neuronal differentiation.

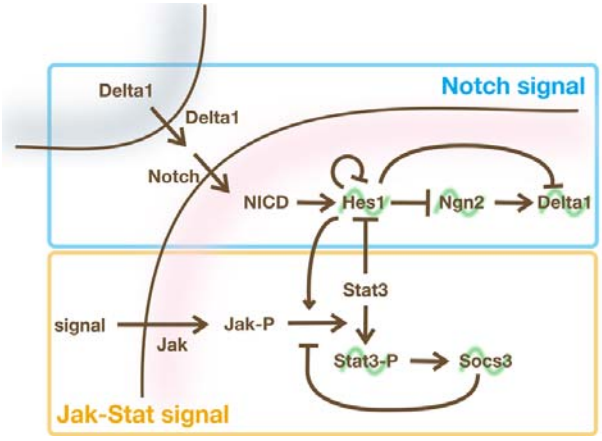

Fig. 6. The oscillator networks in neural progenitor cells. Hes1 expression oscillates due to negative feedback, and Hes1 oscillations drive cyclic expression of proneural genes such as *Ngn2* and Notch ligand genes such as *Delta1*, which in turn activate Notch signaling of neighboring cells. Formation of phosphorylated Stat3 (Stat3-P) and expression of Socs3 may also oscillate due to negative feedback. Hes1 oscillation and Stat3-Socs3 oscillations seem to depend on each other.

destabilized luciferase reporter (Masamizu et al., 2006) revealed that *Hes1* expression oscillates in neural progenitor cells in the developing nervous system (Fig. 6) (Shimojo et al., 2008). Like Hes7, Hes1 oscillation is regulated by negative feedback (Hirata et al., 2002). Interestingly, *Ngn2* and *Delta1* expression levels exhibit an inverse correlation to Hes1 expression levels, raising the possibility that *Ngn2* and *Delta1* expression also oscillate in neural progenitor cells. Real-time imaging revealed that *Ngn2* and *Delta1* expression are indeed oscillatory in neural progenitor cells (Fig. 6) but are sustained (non-oscillatory) in differentiating neurons, which lose Hes1 expression (Shimojo et al., 2008). Sustained repression of Hes1 by inhibition of Notch signaling leads to sustained up-regulation of Ngn2 and Delta1 and thereby to neuronal differentiation, indicating that Hes1 oscillation induces *Ngn2* and *Delta1* oscillations. It seems that Ngn2 can induce neuronal differentiation when the expression is sustained but not when the expression is oscillatory. Ngn2 is known to induce neuronal differentiation by up-regulating many downstream genes, but these downstream genes are likely to be expressed only when Ngn2 is expressed persistently. When Ngn2 expression oscillates, only subsets of downstream genes such as *Delta1* seems to be selectively expressed. Thus, it is likely that Ngn2 oscillation is advantageous for maintenance of neural progenitor cells by mutual activation of Notch signaling through periodic induction of Delta1. These results suggest that *Ngn2* regulates two opposite events depending on the expression mode: maintenance of neural progenitor cells when expressed cyclically and induction of neuronal differentiation when expressed persistently (Kageyama et al., 2008).

Unlike the Hes7 oscillation in the PSM, Hes1 oscillation in the developing nervous system is very unstable: the amplitude and period are variable from cycle to cycle and from cell to cell (Shimojo et al., 2008). We speculate that when the Hes1 level is high during G1 phase, these cells are resistant to neuronal differentiation but not when the Hes1 level is low during G1 phase. Thus, it is possible that neural progenitor cells respond differently to the same environmental conditions depending on the Hes1 level, thereby contributing to generation of diversity in the developing brain.

Oscillatory versus non-oscillatory expression of Hes1

The neural tube is partitioned into many compartments by boundaries. Boundary cells proliferate slowly and usually do not give rise to neurons, unlike neural progenitor cells in compartments. Interestingly, Hes1 expression seems to be non-oscillatory in boundary cells (Baek et al., 2006). Non-oscillatory Hes1 expression not only leads to G1 phase retardation but also inhibits neuronal differentiation by constitutively repressing proneural gene expression. Thus, it is likely that oscillatory versus non-oscillatory Hes1 expression regulates compartment versus boundary cell characteristics.

A precise mechanism of how oscillatory versus non-oscillatory expression of Hes1 is regulated is currently unknown, but Jak-Stat signaling seems to be involved because a Jak inhibitor, AG490, stops Hes1 oscillation in fibroblasts and neural progenitor cells (Shimojo et al., 2008). AG490 stabilizes Hes1 protein in fibroblasts (Yoshiura et al., 2007). As stated above, the instability of gene products is essential for oscillatory expression, and thus it is likely that Jak regulates Hes1 oscillation by inducing the instability of Hes1 protein. Conversely, activation of Stat3 depends on Hes1 protein, which facilitates interaction of Jak and Stat (Kamakura et al., 2004). Interestingly, in fibroblasts, Jak-dependent activation of Stat3 induces expression of Socs3, which inactivates Stat3, thus forming negative feedback. This negative feedback leads to oscillation in activities in Jak-Stat signaling (Yoshiura et al., 2007). Thus, it is likely that oscillations in Notch-Hes1 and Jak-Stat signaling are coupled with and depend on each other (Fig. 6).

Other systems with ultradian rhythms

In the PSM, in addition to Hes7 and Notch and Fgf signaling effectors, expression of Wnt signaling molecules also oscillates (Aulehla and Herrmann, 2004; Dequéant, et al., 2006). In neural progenitor cells, expression of *Hes1*, proneural genes and Notch ligand genes oscillates. It was shown that Jak-Stat3 and Smad1-Smad6 display oscillatory expression by negative feedback in fibroblasts (Yoshiura et al., 2007). These results indicate that oscillatory expression is a general phenomenon observed commonly in many biological events. In agreement with

this idea, NF-κB and p53 signaling also display oscillatory responses.

NF-κB factors control diverse signaling responses that mediate inflammation, cell growth and survival. Activation of this signaling leads to degradation of IκB and transport of NF-κB from the cytoplasm to the nucleus. NF-κB then induces expression of IκB, which sequesters NF-κB in the cytoplasm. As a result, the localization of NF-κB oscillates between the nucleus and the cytoplasm (N-C oscillations) with a period of about 100 min (Hoffmann et al., 2002; Nelson et al., 2004). It is suggested that difference in N-C oscillation frequency and persistence is involved in differential cellular responses.

The tumor suppressor p53 regulates DNA repair and apoptosis after DNA damage, and it has been shown that p53 is expressed cyclically during this process. p53 induces expression of Mdm2, which leads to degradation of p53. This negative feedback again generates oscillatory responses in p53 expression with a period of several hours (Bar-Or et al., 2000; Lahav et al., 2004). Interestingly, with stronger stimulation, more pulses of p53 oscillations occur, but the amplitude and period are relatively constant at the single cell level (Lahav et al., 2004). Thus, the p53 response is rather digital (the number of pulses) than analog in individual cells, and the number of p53 pulses could decide which way to go, DNA repair or apoptosis.

Oscillatory expression seems to be a general feature for many signaling pathways, although the expression modes of the downstream target genes are not well characterized. If the downstream target gene products are stable, they do not respond in an oscillatory manner, and the expression may be maintained within a certain range or gradually up-regulated due to accumulation. In the latter case, when the expression level reaches a certain value, the next event could happen, indicating that 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.

ACKNOWLEDGMENTS

This work was supported by the Genome Network Project and the Grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Uehara Memorial Foundation. Y.N. and H.S. were supported by the 21st Century COE Program of the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Research Fellowship of the Japan Society for the Promotion of Science for Young Scientist.

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