RESEARCH COMMUNICATION

Different types of oscillations in Notch and Fgf signaling regulate the spatiotemporal periodicity of somitogenesis

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Somitogenesis is controlled by cyclic genes such as Notch effectors and by the wave front established by morphogens such as Fgf8, but the precise mechanism of how these factors are coordinated remains to be determined. Here, we show that effectors of Notch and Fgf pathways oscillate in different dynamics and that oscillations in Notch signaling generate alternating phase shift, thereby periodically segregating a group of synchronized cells, whereas oscillations in Fgf signaling released these synchronized cells for somitogenesis at the same time. These results suggest that Notch oscillators define the prospective somite region, while Fgf oscillators regulate the pace of segmentation.

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Somite formation occurs periodically by segmentation and maturation of a block of cells in the anterior presomitic mesoderm (PSM) (Dequéant and Pourquié 2008). It is thought that the pace of segmentation depends on the clock controlled by cyclic genes such as Notch signaling molecules, while the timing of maturation depends on the wave front established by morphogens such as Fgf8 (Dubrulle et al. 2001; Sawada et al. 2001; Lewis et al. 2009). However, Notch signaling oscillations become slower than the pace of segmentation as the oscillations are propagated anteriorly (Palmeirim et al. 1997; Giudicelli et al. 2007), raising the question of whether such a slowing oscillator regulates the segmentation pace. Furthermore, Fgf signaling seems to sweep back at a steady speed as the PSM grows (Dubrulle and Pourquié 2004; Aulehla and Pourquié 2010), raising another question of whether the release from Fgf signaling occurs at

different times between the anterior and posterior cells even in the same prospective somites.

In the mouse PSM, *Hes7* is expressed in an oscillatory manner and induces oscillatory expression of *Lunatic fringe* (*Lfng*), a modulator of Notch signaling (Bessho et al. 2001; Dale et al. 2003; Serth et al. 2003). *Lfng* oscillations in turn lead to cyclic formation of the Notch intracellular domain (NICD) (Huppert et al. 2005; Morimoto et al. 2005), an active form of Notch, which then periodically induces expression of *Mesp2*, an essential gene for the segmentation and rostro-caudal patterning of each somite (Saga et al. 1997). *Mesp2* expression depends on NICD and *Tbx6* and occurs after the release from Fgf and Wnt signaling in the whole S-1 region, a group of cells that forms a prospective somite (Evrard et al. 1998; Zhang and Gridley 1998; Aulehla et al. 2008; Oginuma et al. 2008, 2010). High-resolution in situ hybridization demonstrated that S-1 cells synchronously exhibit nuclear dots of *Mesp2* signals, indicating synchronous initiation of *Mesp2* transcription in the whole S-1 region (Oginuma et al. 2008, 2010). In *Lfng*-null mice, which have segmentation defects (Evrard et al. 1998; Zhang and Gridley 1998), *Mesp2* expression becomes randomized in S-1 cells, displaying a salt-and-pepper pattern (Oginuma et al. 2010). These results suggest that synchronous *Mesp2* expression in S-1 cells is important for somite formation. However, how slowing Notch signaling oscillators and steadily regressing Fgf and Wnt signaling regulate periodic and synchronous *Mesp2* expression in S-1 cells remains to be determined.

Here we found that Notch and Fgf signaling effectors oscillate with different dynamics and that oscillations in Notch signaling periodically segregate a group of synchronized cells, whereas oscillations in Fgf signaling release these synchronized cells for somitogenesis at the same time. These results suggest that Notch oscillators define the prospective somite region, while Fgf oscillators regulate the pace of segmentation, thereby linking the clock and the wave front.

Results and Discussion

Oscillations in Notch and Fgf pathways show different dynamics

To understand the dynamics of oscillators, we first examined the spatiotemporal relationship between *Hes7* and NICD by immunohistochemistry. When *Hes7* protein was expressed in the middle PSM (Fig. 1A,C), NICD was expressed in S-1 and the posterior PSM (phase I) (Fig. 1B,C). During this phase, *Hes7* protein was also expressed by subsets of cells in the posterior end of the PSM (Fig. 1A,C). When *Hes7* protein expression moved to the anterior PSM, NICD expression in the posterior PSM and S-1 also moved anteriorly (phase II) (Figure 1G–I). *Hes7* expression further moved to S-1, and new *Hes7* protein expression was initiated in the posterior PSM (phase III) (Fig. 1M–O). During this phase, NICD expression moved to S0 and S-2 (Fig. 1N,O). Thus, NICD and *Hes7* protein were expressed in a mutually exclusive manner during all three phases, except for the posterior end of the PSM. In addition, both the *Hes7* and NICD expression domains were narrowed as they moved anteriorly, and formed two regions with alternating phase

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Figure 1. Dynamic expression of Notch and Fgf signaling molecules in the PSM. Immunostaining of the PSM sections of E10.5 wild-type (A–F, phase I, $n = 4$; G–L, phase II, $n = 5$; M–R, phase III, $n = 4$), and *Hes7* knockout (KO) (S–X, $n = 8$) embryos. Each row shows the same embryo. Double-staining of Hes7 and NICD and of pERK and Dusp4 was performed in the same sections. NICD and pERK oscillations showed different dynamics between each other in the wild type. *Hes7*-null mice showed a single pattern of NICD, pERK, and Dusp4 expression, indicating that NICD, pERK, and Dusp4 oscillations depend on *Hes7*.

shifts: $Hes7^+NICD^-$ (S-1) and $Hes7^-NICD^+$ (S-2) (Fig. 1M–O). In *Hes7*-null mice, NICD was expressed steadily in the PSM (Fig. 1S–U), indicating that dynamic NICD expression depends on *Hes7*.

We next examined the spatiotemporal profiles of Fgf signaling molecules. *Hes7* induces oscillatory expression of *Dusp4*, an inhibitor of Fgf signaling (Niwa et al. 2007), which in turn leads to cyclic formation of the Fgf effector phosphorylated ERK (pERK) (Sawada et al. 2001; Delfini et al. 2005; Niwa et al. 2007). During phase I, Dusp4 was expressed in the posterior to middle PSM, while pERK expression was low (Fig. 1D–F). During phase II, Dusp4 expression was down-regulated, while pERK expression was up-regulated in the posterior to middle PSM (Fig. 1J–L). During phase III, Dusp4 expression was somewhat up-regulated, while pERK expression was further up-regulated in the posterior to S-2 region and also appeared in S0 (Fig. 1P–R). Thus, pERK expression occurred in an oscillatory manner in the posterior to S-2 region. *Hes7*-null mice showed a single pattern of pERK and Dusp4 expression (Fig. 1V–X), indicating that pERK and Dusp4 oscillations depend on *Hes7*. These results indicated that *Hes7*-dependent NICD and pERK oscillations showed different dynamics between each other: NICD oscillation displayed a band propagation pattern from the posterior to S0, while pERK oscillation displayed an on-off pattern in the posterior to S-2.

Notch signaling is required for segregation of synchronized regions

The expression patterns were further examined at a single-cell level. NICD and Hes7 protein expression were clearly separated in the middle to anterior PSM (Fig. 2A,B,B',D,E,E',G,H,H'). In contrast, in the posterior end

of the PSM, $NICD^+$ cells and $Hes7^+$ cells were intermingled, and subsets of cells even coexpressed NICD and Hes7 in all three phases (Fig. 2A,C,D,F,G,I). Thus, synchronization and segregation of Hes7 and NICD oscillations were not complete in the posterior end but became evident in the middle to anterior regions.

We next asked how the synchronization is regulated in the PSM. It was proposed previously that Notch signaling is involved in synchronization of oscillations in zebrafish (Jiang et al. 2000; Horikawa et al. 2006; Riedel-Kruse et al. 2007). To address this issue, we examined *Lfng*-null mice, where oscillations in Notch signaling are disrupted (Morimoto et al. 2005). In the posterior end of the PSM of *Lfng*-null mice, $Hes7^-$ and NICD-expressing cells were intermingled, and some cells coexpressed Hes7 and NICD (Fig. 2J,L). Thus, the expression pattern was not significantly affected compared with the wild type in the posterior end of the PSM (Fig. 2C,F,I,L). However, Hes7 and NICD expression domains were not clearly segregated in either the S0 or S-1 region of *Lfng*-null mice (Fig. 2J,K,K'): $Hes7^+$ cells and $NICD^+$ cells were scattered throughout the PSM and were intermingled with each other (Fig. 2J,K). Some cells even coexpressed Hes7 and NICD, similar to what occurred in the posterior end of the PSM (Fig. 2J–L). These results indicated that *Lfng* regulates synchronization and segregation of Hes7 and NICD oscillations, thereby forming

alternating phase-shifting regions: S-1 and S-2. It was shown previously that, in *Lfng*-null mice, NICD expression is nonoscillatory (Morimoto et al. 2005), whereas another study indicated that it is oscillatory (Ferjentsik et al. 2009). This discrepancy remains to be clarified, but our results indicated that synchronized NICD oscillation was severely impaired in *Lfng*-null mice (Fig. 2J–L).

We further analyzed the oscillation dynamics by time-lapse imaging with *Hes7* promoter-driven Ubluc reporter mice (Masamizu et al. 2006; Takashima et al. 2011). In the wild type, the reporter expression oscillated with high amplitudes (Supplemental Movie S1), and, from this imaging, the spatiotemporal profiles were produced (Supplemental Figs. S1, S2A,G). In *Hes7*-null mice, the reporter expression did not oscillate, leading to severe segmentation defects (Supplemental Fig. S2C,E,G; Supplemental Movie S3). In *Lfng*-null mice, the reporter expression oscillated with the same periodicity as that of the control but showed ambiguous and broader patterns (Supplemental Fig. S2B,G; Supplemental Movie S2). *Uncx4.1* expression was also ambiguously segmented in these mice compared with the control (Supplemental Fig. S2D,E). These results indicated that *Lfng* is required for narrowing and clear segregation of the $Hes7^+$ and $NICD^+$ domains, leading to robust oscillatory expression of Hes7 and NICD.

pERK oscillations release the whole S-1 region for differentiation at the same time

We next examined the relationship between NICD and pERK. During phases II and III, the posterior NICD expression moved from the middle to S-2 region, while pERK was expressed from the posterior to S-2 region (Fig. 3A–D), indicating that the posterior NICD domain was

Figure 2. *Lfng*-dependent synchronization and segregation of Hes7 and NICD expression. (A–I) Immunostaining of Hes7 and NICD in the PSM sections of E10.5 wild-type embryos (A,D,G). Boxed regions are enlarged in B, C, E, F, H, and I. (B,B',E,E',H,H') Note that, in the anterior regions, Hes7 and NICD expression were well segregated, and that expression of each factor was well synchronized between neighboring cells. (C,F,I) In contrast, in the posterior end, Hes7- and NICD-expressing cells were intermingled, and many cells coexpressed Hes7 and NICD, indicating that Hes7 and NICD expression are not synchronized or segregated ($n = 16$). (J–L) Immunostaining of the PSM section of an E10.5 *Lfng*-null embryo. Hes7 and NICD expression are not well segregated in the anterior (K) and posterior (L) regions ($n = 5$).

covered by the pERK domain. However, during phase I, when S-2 became S-1 after segmentation, pERK expression was down-regulated and the whole NICD domain in S-1 was freed from pERK (Fig. 3E,F). It was reported previously that *Mesp2* expression is synchronously initiated in S-1 cells during this period (Oginuma et al. 2008, 2010). Because *Mesp2* expression is known to be repressed by Fgf signaling (Oginuma et al. 2008), the time when the NICD domain in S-1 is freed from pERK is likely to coincide with the onset of *Mesp2* expression.

To directly test this idea, we performed time-lapse imaging of *Mesp2* expression and Fgf-ERK signaling activity by using red (SLR) and green (ELuc) luciferase reporters (Nakajima et al. 2004, 2010). Because both luciferase proteins are too stable to monitor oscillatory expression, they were destabilized by ubiquitination (UbSLR and UbELuc). *Mesp2* transcription was monitored by *Mesp2* promoter-driven UbELuc (*Mesp2*-UbELuc), while Fgf-ERK signaling activity was monitored by *Dusp4* promoter-driven UbSLR (*Dusp4*-UbSLR). The expression from the *Dusp4*-UbSLR reporter closely mimicked pERK expression in the PSM (Supplemental Fig. S3A–E). Transgenic mice carrying the *Mesp2*-UbELuc and *Dusp4*-UbSLR re-

porters were generated, and explant cultures of the posterior half of embryos at embryonic day 10.5 (E10.5) were performed for red and green bioluminescence imaging (Fig. 3G, Supplemental Movie S4). During phases II and III, Fgf-ERK signaling (Fig. 3G, red) was active from the posterior to the S-2 region (Fig. 3G, frames 1–3), although the activity gradually decreased. After segmentation, Fgf-ERK signaling was mostly negative, and *Mesp2* expression (Fig. 3G, green) was initiated in the S-1 region (Fig. 3G, frame 4, bottom arrow indicates a newly formed boundary, while a broken line shows a prospective boundary). During phase I, *Mesp2* expression occurred in the whole S-1 region (Fig. 3G, frames 4–6). During phase II, *Mesp2* expression was reduced to the rostral region of S-1, while Fgf-ERK signaling was gradually up-regulated (Fig. 3G, frames 7,8). These results showed that *Mesp2* expression was initiated in the whole S-1 region just after being freed from Fgf-ERK signaling.

Fgf signaling is required for periodic expression of *Mesp2*

The above results indicated that oscillations in Notch signaling make a group of cells to express NICD synchronously, while oscillations of Fgf-ERK signaling release the NICD⁺ cells from inhibition, thereby enabling all of

Figure 3. Phase shifting between NICD and pERK oscillations and onset of *Mesp2* expression. (A–F) Immunostaining of Hes7 and NICD in the PSM sections of E10.5 wild-type embryos (A, $n = 5$; C, $n = 6$; E, $n = 4$). Boxed regions are enlarged in B, D, and F. (G) Time-lapse imaging of *Dusp4*-UbSLR (red) and *Mesp2*-UbELuc (green) reporter expression in the E10.5 PSM ($n = 3$). Note the spatiotemporal coincidence of *Dusp4* disappearance and *Mesp2* onset (just below the broken line in frame 4).

the S-1 cells to express *Mesp2* at the same time. We next examined how *Mesp2* expression is affected without oscillators. Time-lapse imaging analysis showed that, in the wild type, *Mesp2* expression occurred periodically in S-1 (Fig. 4A,A',E; Supplemental Fig. S4A), as reported previously (Saga et al. 1997). In *Hes7*-null mice, the pERK expression domain did not oscillate, but regressed at a steady speed (Fig. 1V–X), which was also confirmed by the time-lapse imaging of the Dusp4-UbSLR reporter expression (Supplemental Fig. S3F). In these mice, *Mesp2* expression also did not occur periodically, but regressed at a steady speed (Fig. 4B,B'; Supplemental Fig. S4B). Thus, in *Hes7*-null mice, the onset of *Mesp2* expression occurred at different times between the anterior and posterior cells even in the same prospective somites, raising the possibility that desynchronized onset of *Mesp2* expression leads to segmentation and patterning defects. These results indicated that oscillations in Fgf-ERK signaling are required for periodic expression of *Mesp2* in the whole S-1 region.

We further analyzed the effect of oscillations in Fgf-ERK signaling on *Mesp2* expression. We first attempted continuous inactivation of Fgf signaling. However, in *Fgfr1*-null mice, oscillations of both *Hes7* and *Lfng* are lost (Niwa et al. 2007), and therefore the observed defects could be due to loss of oscillations in both Notch and Fgf signaling. We and others showed previously that treatment with Fgf signaling inhibitors rapidly blocked Fgf signaling but only slowly affected Notch signaling (Niwa et al. 2007; Wahl et al. 2007). Thus, we examined the early effect of SU5402, an inhibitor of Fgf signaling, on *Mesp2* expression. SU5402 treatment completely abolished the Dusp4-UbSLR reporter expression (Supplemental Fig. S3G), suggesting that Fgf-ERK activities were lost. Time-lapse imaging analysis demonstrated that, without Fgf-ERK activities, *Mesp2* expression occurred continuously and prematurely (Fig. 4C,C', the third signal appeared prematurely and was fused with the second), compared with the wild type (Fig. 4A,A'). In the wild type, *Mesp2* expression was initiated in S-1 just after segmentation (Fig. 4E, control, frames 43,61), whereas, in SU5402-treated PSM, the new *Mesp2* expression occurred prematurely in S-2 and the further posterior region before segmentation (Fig. 4F, +SU5402, frame 28, white arrow, note that the next segmentation is indicated by the lowest black arrow in frame 37). We next attempted continuous activation of Fgf signaling by treatment of the Dusp inhibitor BCI (Molina et al. 2009). BCI treatment led to steady expression of the Dusp4-UbSLR reporter (Supplemental Fig. S3H), suggesting that Fgf-ERK activities were steadily active. Time-lapse imaging analysis showed that *Mesp2* expression was not periodic but regressed steadily in the presence of BCI (Fig. 4D,D',G), suggesting that *Mesp2* expression occurred in the anterior PSM after steady regression of Fgf-ERK activities. These results support the idea that Fgf-ERK-dependent oscillations regulate the periodic onset of *Mesp2* expression in S-1.

It has been shown that Notch signaling is required for synchronization of oscillations between PSM cells in zebrafish (Jiang et al. 2000; Horikawa et al. 2006; Riedel-Kruse et al. 2007), and our study agreed with this idea but showed that *Lfng* is required for synchronization in mice. *Hes7* and NICD oscillations become ~1.5-fold slower in the anterior PSM than in the posterior, and it is likely that these slowing oscillations lead to narrowing and segrega-

Figure 4. Fgf signaling-dependent periodic expression of *Mesp2*. Time-lapse imaging of *Mesp2* expression with *Mesp2*-UbELuc reporter mice. *Mesp2* expression occurred periodically in the control (A,A', $n = 5$), whereas it occurred steadily in *Hes7* knockout (KO) (B,B', $n = 4$) embryos. (C,C') In explants treated with the Fgf inhibitor SU5402 (30 μ M), *Mesp2* expression occurred precociously ($n = 3$). (D,D') In explants treated with the Dusp inhibitor BCI (2 μ M), *Mesp2* expression regressed steadily ($n = 5$). The filmstrips of A, C, and D are shown in E–G, respectively. Somite boundaries are indicated by black arrows. Prospective somite boundaries are shown by broken lines. Dominant positions of *Mesp2* expression are indicated by white arrows.

tion of the synchronized regions (mathematical modeling; Supplemental Figs. S5–S8). If these oscillations do not slow in the anterior PSM, the somite sizes are likely to be severely impaired (mathematical modeling; Supplemental Fig. S9). Thus, the slowing oscillations in Notch signaling are important to define the prospective somite region. Oscillations in Fgf signaling then periodically free the NICD domain from inhibition, thereby inducing *Mesp2* expression in the whole S-1 region at the same time (Fig. 5). These features agree well with the traditional concept of a “clock and wave front model” (Cooke and Zeeman 1976): Notch oscillators constitute the clock, while Fgf oscillators constitute the rippled wave front. Importantly, these oscillators are coupled by *Hes7* in the posterior PSM but are phase-shifting in the anterior PSM. Our results indicated that Notch and Fgf oscillators regulate the periodicity in space and time, respectively, and that *Hes7* integrates these oscillators, thereby linking the clock and the wave front.

Materials and methods

Immunohistochemistry

Whole-mount immunohistochemistry was performed as described previously (Bessho et al. 2003). Immunostaining was performed with 4- μ m paraffin sections of E10.5 embryos as described previously with a minor modification (Morimoto et al. 2005). Antibodies against *Hes7* (Bessho et al. 2003), Dusp4 (Santa Cruz Biotechnology), pERK, and NICD (Cell

Figure 5. Schematic representation of dynamic expression of Hes7, NICD, pERK, and *Mesp2*. (Left three panels) The posterior NICD domain moves anteriorly and narrows, while the pERK domain expands anteriorly, covering the NICD domain. (Right panel) After segmentation, pERK expression is down-regulated, and NICD now induces *Mesp2* expression in the S-1 region. Thus, oscillations in Notch signaling periodically segregate a group of synchronized cells, and oscillations in Fgf signaling release these synchronized cells for somitogenesis at the same time.

Signaling Technology) were used. High-power pictures were made by stacking several sequential confocal images (total, 5 μ m).

Generation of luminescence reporter mice and PSM culture

Generation of UbLuc reporter mice and a culture condition were described previously (Bessho et al. 2001; Masamizu et al. 2006). For *Mesp2*-UbELuc and *Dusp4*-UbSLR mice, the regions from ATG to the stop codon of the *Mesp2* and *Dusp4* genomes were replaced with ubiquitinated green and red luciferase cDNAs by using BAC recombineering. The fragment containing 7.3 kb of the *Mesp2* promoter or 8.2 kb of the *Dusp4* promoter as well as 0.5 kb of the 3' untranslated region (UTR) was retrieved into pBS vector. Genotypes were determined by PCR using the primers 5'-CAGA GTCTACCTCCGCCAACATC-3' and 5'-CAGGTACTTGAGTGAGACACCC-3' for ELuc and 5'-TTCCACCACGCCTTCGACTGTT-3' and 5'-CACCTGGTAGCCCTTGACTTGAT-3' for SLR.

Time-lapse imaging of luminescence and image analysis

Time-lapse imaging of luminescence was captured as was described previously (Masamizu et al. 2006). We used ImageJ software for time-lapse imaging analysis. To make spatiotemporal profiles, we first applied a median filter to Hes7-UbLuc stack images and a spike-noise filter to *Mesp2*-UbELuc and *Dusp4*-UbSLR stack images to remove the cosmic rays. Then, we got the resliced stack images in the anterior-posterior axis with average or median values and arranged them from left to right in the temporal order. To obtain clear dynamic *Dusp4*-UbSLR expression in dual-color images, we showed only the modified values where minimal values were subtracted from filtered images in each position.

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