RESEARCH COMMUNICATION

Zebrafish Hairy/Enhancer of split protein links FGF signaling to cyclic gene expression in the periodic segmentation of somites

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FGF signaling pathways have been implicated in the establishment of proper periodicity of vertebrate somites. Here, we show evidence that a Hes6-related hairy/Enhancer of split-related gene, her13.2, links FGF signaling to the Notch-regulated oscillation machinery in zebrafish. Expression of her13.2 is induced by FGF-soaked beads and decreased by an FGF signaling inhibitor. her13.2 is required for periodic repression of the Notch-regulated genes her1 and her7, and for proper somite segmentation. Furthermore, Her13.2 augments autorepression of her1 in association with Her1 protein. Therefore, FGF signaling appears to maintain the oscillation machinery by supplying a binding partner, Her13.2, for Her1.

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Somites are the morphologically distinct segmental units that are transiently formed during early vertebrate development and subsequently give rise to metameric and fundamental structures such as the vertebrae of the axial skeleton, their associated muscles, and tendons. The somites are subdivided from the anterior end of the unsegmented paraxial mesoderm, called the presomitic mesoderm (PSM), and sequentially generated in an anterior to posterior direction in a rhythmic fashion at regular spatiotemporal intervals. The molecular mechanism underlying the periodical formation of somites is coupled to an internal oscillator, referred to as the “segmentation clock”, which has been evidenced by the cyclic expression of genes in the PSM (Palmeirim et al. 1997; Maroto and Pourquie 2001). Most genes that exhibit a cyclic expression pattern in the PSM are involved in the Notch signaling pathway (Bessho and Kageyama 2003). Various hairy/Enhancer of split (Espl)-related basic helix-loop–helix (bHLH) genes [hairy-1, hairy-2, and Hey2 in the chicken; Hes1, Hes5, Hes7, and Hey2 in the mouse; her1 and her7 in the zebrafish] that are transcriptional targets of the Notch signaling are expressed in a dynamic pattern of stripes across the PSM in a posterior to anterior direction. In addition, Lunatic fringe, encoding a glycosyltransferase that modulates the Notch signaling, has been shown to be required for the proper somite segmentation in mice (Hrabe de Angelis et al. 1997; Kusumi et al. 1998; Bessho et al. 2001) and zebrafish (Takke and Campos Ortega 1999; Holley et al. 2000, 2002; Henry et al. 2002; Oates and Ho 2002) by mutant analysis or morpholino oligo (MO)-mediated gene-knockdown experiments. Furthermore, the cyclic expression of Hes7, her1, and her7 requires their own activity, suggesting that a negative feedback loop involving these genes is a critical component of the oscillation machinery (Oates and Ho 2002; Bessho et al. 2003).

The oscillating and anteriorly propagating wave of gene expression, which is maintained in the posterior PSM, becomes fixed to cause segmentation in the anterior PSM. Activity gradients of signaling molecules, including fibroblast growth factor (FGF), Wnt, and retinoic acid are proposed to regulate the differentiation of PSM cells along the anteroposterior axis from a state permitting the oscillating gene expression to a state driving the segmentation program (Dubrulle et al. 2001; Sawada et al. 2001; Aulehla et al. 2003; Moreno and Kintner 2004). For instance, FGF signaling is the highest at the posterior end of the PSM with a gradual decrease toward the anterior, suggesting a role for FGF signaling in maintaining the characteristics of the posterior PSM cells (Dubrulle et al. 2001; Sawada et al. 2001). In fact, overexpression of FGF8 in the entire PSM of chick embryos causes an increase in the expression of Brachyury in the posterior PSM and suppresses morphological segmentation (Dubrulle et al. 2001). Furthermore, inhibition or inhibition of FGF signaling results in the formation of smaller or larger somites, respectively. Thus, FGF signaling appears to maintain the posterior characteristics, which allows the cyclic gene expression, and the level of FGF activity regulates the transition of the posterior PSM cells into segmental tissues and determines the position of the segment boundaries. However, the molecular mechanism by which FGF signaling permits the cyclic gene expression in the posterior PSM has been totally unknown.

Here we focused on a zebrafish hairy/Espl gene,
her13.2, which is expressed in the posterior PSM. Our results indicate that FGF signaling allows the cyclic gene expression by inducing her13.2 in the posterior PSM.

Results and Discussion

To gain a better insight into the molecular mechanism underlying the segmentation process, we identified genes specifically expressed in the PSM by an in situ hybridization screening of mRNA expressed in the PSM to tailbud region of zebrafish embryos (18–21-somite stages). Among these genes, her13.2, coding for a bHLH protein closely related to mammalian Hes6 (Bae et al. 2000; Koyano-Nakagawa et al. 2000; Sieger et al. 2004), exhibited posterior-to-anterior decrease in expression, analogous to the FGF signaling gradient, in the posterior PSM and tailbud. Expression of her13.2 was first observed in the blastoderm margin at the shield stage (Fig. 1A), and then became restricted to the posterior region at the tailbud stage, being excluded from the axial mesoderm [Fig. 1B]. As segmentation proceeded, the transcripts became specifically localized to the posterior PSM and tailbud [Fig. 1C–F]. Expression of her13.2 was strong at the tailbud and gradually decreased along the PSM [Fig. 1D]. In contrast to other her genes, e.g., her1 and her7, which are known to exhibit oscillatory expression in the PSM and tailbud (Holley et al. 2000; Sawada et al. 2000; Henry et al. 2002; Oates and Ho 2002; Gajewski et al. 2003), her13.2 is unique for its nonscillatory expression.

Since the graded axial distribution of her13.2 mRNA in the posterior PSM resembled that of FGF activity, we examined whether FGF signaling was involved in the expression of her13.2. With a brief treatment of embryos with SU5402, an FGF receptor kinase inhibitor (Mohammadi et al. 1997), expression of her13.2 in the PSM was drastically reduced, whereas DMSO-treated control embryos maintained the normal expression of her13.2 (Fig. 2A,B). Conversely, implantation of an mFGF8b-soaked bead into the anterior PSM caused anterior expansion of the expression domain of her13.2, whereas a bovine serum albumin (BSA)-soaked control bead did not affect her13.2 expression (Fig. 2C,D). On the other hand, Notch signaling, which is known to activate hairy/Espl-related genes showing oscillatory expression in the PSM (Bessho and Kageyama 2003), did not affect the expression of her13.2 in the PSM. Expression of her13.2 in aei/deltaD−/− embryos, as well as in embryos injected with mRNA encoding the intracellular domain of notch1a, which can activate the Notch signaling constitutively (Takke and Campos-Ortega 1999), appeared indistinguishable from that in normal embryos (Fig. 2E–G). Furthermore, expression of her13.2 was not significantly altered in fss/tbx24 mutant embryos (Fig. 2H). These results indicate that her13.2 functions downstream of FGF signaling in the PSM, but is not under direct regulation by Notch signaling, or Tbx24 transcription factor, in contrast to other hairy/Espl-related genes.
antisense MOs specific for development, we performed antisense MO-mediated experiments. Lateral views of her13.2 MO-injected and Smis-her13.2 MO-injected embryos at the 12-somite stage. In her13.2 MO-injected embryos, the boundaries of somites are disrupted after the proper segmentation of the first eight somites, whereas no apparent defect is seen in Smis-her13.2 MO-injected embryos. Injected embryos were incubated at 23.5°C after the dome stage (4.5 hpl), because this segmental defect was more prominent after this stage.

To investigate the involvement of her13.2 in somite development, we performed antisense MO-mediated gene-knockdown experiments. Injection of two distinct antisense MOs specific for her13.2 resulted in disruption of the regular segmentation of somites posterior to the first seven to nine somites (Fig. 3). This phenotype closely resembled the phenotypes of previously identified segmentation mutants, such as after eight, deadly seven, and mind bomb (van Eeden et al. 1996). In contrast, MOs carrying five base substitutions in the her13.2-specific MOs caused no such defect. Coinjection of variant her13.2 mRNA lacking the her13.2 MO-target sequence rescued embryos from this segmental defect in a dose-dependent manner (Supplementary Table 1). Thus, her13.2 is required for the proper segmentation of somites in the posterior trunk. The segmental defect caused by her13.2-specific MOs was confirmed by analysis of the expression of a series of genes characteristically involved in the segmentation process. For instance, deltaC, deltaD, and mesp-b, which are normally expressed in a segmental pattern, were expressed in a scattered pattern in the anterior PSM of her13.2 MO-injected embryos (Fig. 4A–F), as typically observed in embryos defective in the segmentation process (jiang et al. 2000; Sawada et al. 2001; Holley et al. 2002). In contrast, the expression of fss/tbx24, which is normally expressed uniformly in the anterior and intermediate PSM (Nikaido et al. 2002), was not affected (Fig. 4G,H). Rostrocaudal polarity of somites was also perturbed in her13.2 MO-injected embryos. Expression of myod, which is normally confined to the posterior region of somites (Weinberg et al. 1996), was expanded (Fig. 4I,J), whereas that of paraxial protocadherin (papc), which is normally expressed in the PSM and anterior region of somites (Yamamoto et al. 1998), was scattered and reduced (Fig. 4K,L). These results indicate that her13.2 function is essential for the process of somite segmentation.

In the process of segmentation, hairy/Espl-related genes her1 and her7 in the zebrafish and Hes7 in the mouse play essential roles in the segmentation machinery, because disruption of these genes results in defects in the segmentation and the oscillatory expression of the cyclic genes (Bessho et al. 2001; Henry et al. 2002; Holley et al. 2002).
et al. 2002; Oates and Ho 2002; Gajewski et al. 2003). These genes also exhibit periodical expression in the posterior PSM, the periodicity of which is generated by a negative feedback loop composed of repression of the genes by their own encoded proteins and break of this repression by ubiquitination-mediated degradation of the proteins (Oates and Ho 2002; Bessho et al. 2003; Hirata et al. 2004). Interestingly, in her13.2 MO-injected embryos, the expression of her1 and her7 was observed in the entire region of the PSM (Fig. 4M), as observed in embryos deficient for her1 and/or her7 (Henry et al. 2002; Holley et al. 2002; Oates and Ho 2002; Gajewski et al. 2003), although the expression level was variable among cells in the PSM of the her13.2-deficient embryos. In contrast, the expression of fgf8, no tail (ntl), and spade tail (spt), which exhibit nonoscillating expression in the posterior PSM, was not significantly affected (Fig. 4Q–V). Thus, her13.2 is required for the cyclic repression of her1 and her7 expression. On the other hand, expression of another cyclic gene, deltaC, was decreased by injecting her13.2-specific MO (Fig. 4A,B), indicating that her13.2 acts differently on the expression of this gene. To determine whether the up-regulated expression of her1 and her7 genes was caused by decreased repression of their transcription or by stabilization of their mRNAs, we examined the distribution of her1 nascent transcripts by using a her1 intron probe. In normal embryos, the segmental expression of nascent her1 transcripts was observed (Fig. 4W), as previously reported (Gajewski et al. 2003). On the other hand, in her13.2 MO-injected embryos, the signal was widespread in the posterior PSM, as was the case when a her1 exon probe was used (Fig. 4X). Thus, her13.2 plays an essential role in cyclic repression of her1 transcription in the posterior PSM.

We next addressed how Her13.2 is involved in repression of the her1 gene transcription by conducting an in vitro luciferase reporter assay. The expression of the luciferase gene under the control of an 8.6-kb her1 promoter is sufficient for the normal expression of the her1 gene in the PSM (Gajewski et al. 2003). This promoter activity was repressed by Her1, but not affected by Her13.2, in 293T cells (Fig. 5A). Under this experimental condition, Her13.2 enhanced the self-repression activity of Her1 [Fig. 5B]. Thus, Her13.2 can act as a transcriptional repressor with Her1, and enhancement of her1 repression by Her13.2 may be required for the periodical repression of her1 in the posterior PSM. To function as a transcriptional repressor, Her13.2 appears to require another bHLH protein as a binding partner, because Her13.2 has a uniquely short loop region within its HLH domain, causing an inability to bind to the target sequences (Bae et al. 2000). Thus, it is possible that Her13.2 may enhance the self-repression of her1 through formation of Her1–Her13.2 heterodimer complex. In fact, an in vitro pulldown assay indicated that Her13.2 protein interacted physically with the Her1 protein (Fig. 5C). These results demonstrate that Her13.2 can strongly repress her1 expression, presumably through complex formation with Her1.

Taken together, our findings indicate that FGF signaling induces the expression of her13.2 in the posterior PSM, and, in turn, Her13.2 protein participates in the periodic repression of the Notch-regulated genes, her1 and her7, presumably through formation of a transcriptional repressor complex with at least one of these Her proteins, Her1. FGF signaling has been shown to maintain posterior PSM cells in a state that allows the oscillatory expression of the cyclic genes, and also to be associated with expression of nonperiodic genes, e.g., fgf8, ntl, and spt (Dubrulle et al. 2001; Sawada et al. 2001), whose expression was not obviously altered in her13.2 MO-injected embryos. Therefore, it is likely that FGF signaling is transmitted through several different molecular pathways in the posterior PSM, and that Her13.2 mediates specifically one of the roles of FGF signaling, the regulation of the cyclic genes. On the other hand, we cannot conclude at present whether her13.2 expression is sufficient for this role of FGF signaling. Overexpression of her13.2 did not give us an answer to this question, because the overexpression resulted in malformation of the presomitic mesoderm, which inhibited the precise analysis of the periodic gene expression (data not shown).

In addition to FGF signaling, Wnt signaling has been indicated to regulate the oscillation machinery (Aulehla et al. 2003). Recently, two groups reported that Wnt signaling directly regulates the expression of Dll1, a component of oscillation machinery in mouse embryos (Galceran et al. 2004; Hofmann et al. 2004). Thus, two molecular systems, which are individually activated by FGF or Wnt signaling, regulate the oscillation machinery. The Wnt may lead to speculation that these two regulatory systems coordinate in the maintenance of the oscillation machinery. However, it is uncertain whether these two regulatory systems function simultaneously in a particular species. In mouse, in contrast to zebrafish, the only structural counterpart of her13.2, Hes6, is not expressed in the PSM (Bae et al. 2000). Furthermore, in the zebrafish, despite a number of findings in the mouse, the involvement of Wnt signaling in the segmentation clock.
remains obscure. Thus, we can also speculate that different species may establish their own ways to transmit the signaling gradient to the segmentation clock. We expect that this issue will be solved by more extensive analysis in the future.

 Materials and methods

Fish
All the experiments except mutant analysis were performed using the TL2 inbred line (Kishimoto et al. 2004). The mutant alleles used in this study are after eight (acev230) and fused somites (fsa230).

Construction of cDNA library, screening, and isolation of her13.2
A cDNA library was constructed with mRNA prepared from the PSM to tailbud region of zebrafish embryos at 18–21-somite stages. For collection of cDNA clones expressed in the PSM and tailbud region, a high-throughput in situ hybridization screening was performed in 96-well plates. The precise procedure for this screening will be described elsewhere. One of the cDNA clones obtained by this screening was a partial cDNA fragment of her13.2. Using this fragment as a probe, we obtained an ∼1.2-kb cDNA from a 15–19 hours post-fertilization (hpf) cDNA library.

Whole-mount in situ hybridization
Whole-mount in situ hybridization was performed as described (Nikaido et al. 1997). For the her1 intron probe, the 1.4-kb genomic region corresponding to the her1 intron was obtained by PCR with the following primers: 5’-GCCGGGTAATGTTAATTTGTGTA-3’ and 5’-TTGACATGATGAAATGAAAAGGAAAAGC-3’, and hybridization was carried out as described (Gajewski et al. 2003).

Antisense MO and mRNA synthesis of her13.2
The sequences of MO used in this study are as follows: her13.2 MO1, 5’-TGCAGTCTAGGCCGCTTAAAGGCG-G3’; 5’-isoher13.2 MO1, 5’-TGCATCCTGAGCCTGGAATGG-3’; her13.2 MO2, 5’-GCCGATAGTGGCGCCGTTCTACCTC-3’; 5’-isoher13.2 MO2, 5’-GGAGTTGGCTGGACGCGTCAACCTTC-3’. The DNA fragment encoding the entire ORF of her13.2 was inserted into pcDNA3 vector. Capped her13.2 mRNA was produced with an mMessage mMachine [Ambion].

SUF402 treatment and bead implantation
These experiments were performed essentially as described (Sawada et al. 2001).

Luciferase reporter assay
Reporter gene pcGL3-her1 was constructed by inserting an 8.6-kb fragment upstream of the translational initiation site of zebrafish her1 into the upstream of firefly luciferase gene in pcGL3 Basic vector (Promega). About 1.0 × 104 293T cells were seeded into each well of a 24-well collagen-coated plate (Iwaki). pcPS2, pcPS2-her1, and pcPS2-her1.3 vector with pcGL3-her1 (100 ng) and pRL-TK (0.2 ng) were transiently introduced into the cells using Lugen (Roche). After incubation for 24 h, the cells were lysed, and the lysates were assayed with a Dual-Luciferase reporter assay system [Promega].

GST pullown assay
To generate GST-Her13.2 and GST-Her1 proteins, the DNA fragment corresponding to the entire amino acid sequence of either her13.2 or her1 was inserted in-frame into pcGST4-3 vector [Amersham]. Amersham fusion proteins were purified on glutathione Sepharose 4B [Amersham]. For synthesis of in vitro labeled proteins, 1 µg of either pcPS2-her13.2 or pcPS2-her1 containing the complete amino acid sequence of her1 was added to a TNT Quick coupled transcription/translation Systems [Promega] in the presence of [35S]S-labeled methionine [Amersham]. In 1 mL of 2% BSA in PBS(−), 5 µL of in vitro translation products was mixed and incubated with 5 µg of either GST, GST-Her13.2, or GST-Her1 for 60 min at 4°C. Following extensive washing, the bound proteins were separated in a 12% SDS-polyacrylamide gel.

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