Dynamic expression and essential functions of Hes7 in somite segmentation

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The basic helix–loop–helix (bHLH) gene Hes7, a putative Notch effector, encodes a transcriptional repressor. Here, we found that Hes7 expression oscillates in 2-h cycles in the presomitic mesoderm (PSM). In Hes7-null mice, somites are not properly segmented and their anterior–posterior polarity is disrupted. As a result, the somite segmentations. In vertebral and rib dermatomes such as vertebrae and ribs are severely disorganized. Importantly, the oscillator and Notch signaling is essential for coordinated somite segmentation (Jouve et al. 2000) and coordinated segmentation of somites: mutations for Notch1, the ligands Delta-like 1 (Dll1) and Dll3, and other regulators such as Presenilin, RBP-J, and lunatic fringe (Lfng) all exhibit defects of somite segmentation [Swiatek et al. 1994; Conlon et al. 1995; Oka et al. 1995; Hrabde Angelis et al. 1997; Jiang et al. 2000; Evrad et al. 1998; Huppert et al. 2000; Koizumi et al. 2001]. Among these molecules, Lfng expression also oscillates in the PSM like chairy1-related genes (Forsberg et al. 1998; McGrew et al. 1998; Aulehla and Johnson 1999), whereas expression of most other Notch pathway genes does not; suggesting that Lfng and chairy1-related genes are the key regulators to convert the temporal component (oscillation) to the spatial component (segmentation). Unlike Lfng, however, the significance of chairy1-related genes in somite segmentation is still obscure because the Hes1 mutation does not affect somitogenesis in mice [Ishibashi et al. 1995; Jouve et al. 2000] and other species lacking chairy1-related genes are not available.

Recently, we isolated a new chairy1-related gene from mouse, named Hes7 [Bessho et al. 2001]. Hes7 encodes a Hes-like transcriptional repressor and its expression is controlled by Notch signaling. Interestingly, Hes7 is expressed in the PSM in a dynamic manner [Bessho et al. 2001], raising the possibility that Hes7 expression is cyclic in the PSM. Here, we found that Hes7 expression oscillates in 2-h cycles in the PSM synchronously with Lfng expression. Strikingly, in mice mutant for Hes7, somites are not properly segmented and their anterior–posterior [A–P] polarity is disrupted. As a result, the somite derivatives, such as vertebrae and ribs, are severely disorganized. Importantly, the oscillator Lfng is expressed continuously throughout the mutant PSM and therefore the oscillating expression is disrupted. These results indicate that Hes7 controls the cyclic expression of Lfng and is essential for somite segmentation.

Results and Discussion

Dynamic expression of Hes7 in the PSM

Hes7 displays various expression patterns in the PSM even at the same developmental stages (Fig. 1A), raising the possibility that Hes7 expression oscillates in the PSM. To investigate this possibility, we compared the expression pattern of Hes7 and the oscillator Lfng in the same embryos. The caudal parts of embryonic day E9.5

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mouse embryos were cut into halves along the midline and the two halves were separated separately to in situ hybridization with Hes7 and Lfng probes [Fig. 1B–D]. It was reported previously that oscillating gene expression pattern is categorized into three distinct stages [Palmeirim et al. 1997]. During stage I, a broad expression domain appears in the posterior PSM like Lfng, although Lfng expression still remained in somite II [Fig. 1B]. During stage II, Hes7 expression moved anteriorly [Fig. 1C] and, during stage III, Hes7 was expressed in somite II like Lfng, but Hes7 expression was extended caudally compared with Lfng expression [Fig. 1D]. These results indicate that, although Lfng expression persists in presumptive somites for a longer period, the main expression domain of Hes7 and Lfng in the PSM is well overlapped, suggesting that expression of these two genes oscillates in the same phases.

To clarify the oscillating expression of Hes7 further, we carried out explant culture experiments. The posterior part of E9.5 mouse embryos was cut along the midline and one half piece was fixed immediately and the other half was cultured for various time before fixation. Both pieces were then hybridized with a Hes7 probe [Fig. 1E–G]. When one half was cultured for 60 or 90 min, the expression patterns of Hes7 were different from those of the uncultured half [Fig. 1E,F]. In contrast, when cultured for 2 h, the explants had a new somite, but the Hes7 expression pattern became very similar to the uncultured pattern [Fig. 1G], indicating that Hes7 expression oscillates in 2-h cycles. Taken together, these results indicate that Hes7 expression oscillates synchronously with Lfng expression in the PSM. In addition, each cycle seems to be linked with generation of one somite.

In chick, the oscillation of chairy1 expression does not require de novo protein synthesis, whereas that of Lfng does [Palmeirim et al. 1997; McGrew et al. 1998]. We next examined whether cyclic expression of Hes7 requires de novo protein synthesis by culturing the caudal parts of mouse embryos in the presence of cycloheximide. Cycloheximide treatment blocked cyclic expression of Hes7 [data not shown], suggesting that Hes7 expression depends on de novo protein synthesis. This result contrasts with that of chairy1, but the molecular mechanism for this difference between Hes7 and chairy1 remains to be determined.

**Generation of Hes7-mutant mice**

To understand the role of Hes7 in somitogenesis, the Hes7 gene was disrupted by homologous recombination in embryonic stem (ES) cells. Most of the coding region of Hes7 was removed and, instead, lacZ gene was placed under the control of the Hes7 promoter [Fig. 2A]. Chimeric mice were generated from the mutant ES cells and bred to produce heterozygous mutant mice. Hes7 heterozygous mutant mice looked normal overall. Forty-three percent of them (51 out of 118), however, had kinked tails [Fig. 2C], suggesting that the dose of Hes7 gene is important for normal tail structure. Intercrossing of the

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**Figure 1.** Dynamic expression of Hes7 in the PSM. (A) Variable expression patterns for Hes7 of E9.5 embryos. (B–D) Comparison of the expression of Hes7 (left) and Lfng (right) in the bisected caudal portions of E9.5 embryos. The expression patterns of two genes were variable, but similar to each other in all three stages (stage I, n = 4; stage II, n = 4; stage III, n = 2). Each pair is dissected from the same embryo. The established somites were stained with the Uncx5.1 probe for spatial alignment. [E–G] Hes7 is differentially expressed after explant culture. The right halves were cultured for 60 min [E], 90 min [F], or 120 min [G], and compared with the left halves, which were fixed immediately. (G) Somite boundaries and a newly formed boundary are indicated by arrows and arrowhead, respectively. Somite -I is indicated by asterisks.

**Figure 2.** Generation of Hes7-null mice. (A) Targeting strategy. The top line shows the structure of the wild-type Hes7 gene and the middle line shows the structure of the targeting vector. Most of the coding region of Hes7 was replaced by IRES–LacZ and PGK–neo [inverted orientation]. The bottom line indicates the resultant disrupted locus. Diptheria toxin gene (DT) was used for negative selection. [E] EcoRV; [S] SacI; [K] KpnI. The positions of 5′-external and 3′-external probes are indicated on top. (B) Southern blot analysis. The 5′-external probe detected 10-kb wild-type and 7-kb mutant bands of SacI-digested genomic DNA. (C) Kinked tails of adult heterozygous mutants. (D) Whole-mount in situ hybridization for Hes7 of wild-type (left) and homozygous-mutant (right) embryos at E9.5. In the mutant embryo, Hes7 expression is completely missing [arrows]. (E) The appearance of heterozygous- [left] and homozygous-mutant [right] neonates. A homozygous mutant has a short trunk and a short tail [arrow].
heterozygous mice generated Hes7 homozygous mutant mice [Fig. 2B], which lacked Hes7 expression [Fig. 2D, arrows]. Hes7-deficient neonates had short trunks and tails, but their head and limbs appeared grossly normal [Fig. 2E]. Most of them died within a few hours after birth, apparently from respiratory failure. The lung did not expand in Hes7-deficient mice, probably because of abnormality of vertebrae and ribs [see Fig. 3F]. A few homozygous mutants, however, survived to adulthood.

Segmentation defects of Hes7-deficient mice

Because the external appearance of Hes7 homozygous neonates suggested malformations of skeletons, we next made skeletal preparations of neonates [Fig. 3A–F]. No abnormality was observed in the skeletons of heterozygous mutant mice except for the tail [Fig. 3A,C,E]. In contrast, in homozygous neonates, severe segmentation defects of axial skeletons were observed [Fig. 3B]. The vertebrae and ribs were abnormally formed [Fig. 3D,F].

Vertebral bodies and neural arches were fused to each other throughout the entire length of the vertebral column, and the total length of vertebrae was significantly short [Fig. 3C,D]. The ribs were fused and bifurcated, and the symmetry of right–left axis was lost. The number of rib pairs was reduced to eight to nine (normally 13), and the thoracic space was severely reduced [Fig. 3E,F]. Sagittal sections of neonates also showed segmentation defects of axial skeletons in homozygous mutants [Fig. 3G,H], such as malformation and fusion of vertebral bodies, intervertebral disks, and neural arches [Fig. 3H]. There was no difference in ossification among neonates of the three genotypes [data not shown], however, indicating that segmentation was primarily affected in Hes7-deficient mice.

To investigate the abnormal segmentation further, we examined the somite formation of Hes7-mutant embryos. In wild-type and heterozygous embryos, a constant size of epithelial somites were formed repeatedly [Fig. 3I], whereas in homozygous embryos, they were irregular in both size and shape and the boundaries between somites were less clear [Fig. 3J, arrow and arrowhead]. In addition, the left–right symmetric somite formation was disrupted in the homozygous embryos [data not shown]. These results indicate that, in Hes7-deficient mice, somite segmentation occurred uncoordinatedly, and it is likely that this abnormal segmentation leads to severe defects of axial skeletons.

Defects of somite patterning in Hes7-deficient embryos

To examine the defects of somite segmentation further, we next analyzed several PSM-expressing genes. Because lacZ was placed under the control of the Hes7 promoter in the mutant allele, we first performed X-gal staining to determine whether the Hes7 promoter activity was affected in Hes7-null mice. In Hes7(−/−) embryos, X-gal staining was observed in several caudal somites as well as in the PSM, probably because the lacZ product is stable [Fig. 4A]. The staining was not restricted to the anterior or posterior half but observed in the whole region of each somite [Fig. 4A, lower left panel]. In a Hes7(−/−) embryo, staining in caudal somites was fused and the boundary between somites I and −I was not clear [Fig. 4A, right panels]. Interestingly, in the Hes7(−/−) embryos, the intensity of X-gal staining was much stronger than in the Hes7(+/−) embryos [Fig. 4A], which cannot be explained by two copies of the lacZ gene, suggesting that the Hes7 promoter activity is up-regulated in the absence of Hes7. Because Hes7 is a transcriptional repressor [Bessho et al. 2001], these data suggest that Hes7 may repress its own expression.

The bHLH gene Paraxis is essential for formation of epithelial somites [Burgess et al. 1996]. Paraxis expression was not changed significantly in the PSM of Hes7-null embryos, although the expression in the mutant somites was fused [Fig. 4B]. This observation is consistent with the above results in that, although irregular, epithelial somites were formed in Hes7 homozygous embryos [Fig. 3J]. Another bHLH gene, pMesogenin, regulates expression of Delta/Notch pathway molecules and controls somitogenesis [Yoon and Wold 2000]. pMesogenin expression was not changed significantly in the PSM of Hes7-null embryos [Fig. 4C]. Expression of the bHLH gene Mesp2, which specifies the anterior half of each somite [Saga et al. 1997], was reduced slightly com-
pared with the wild type but remained in the anterior PSM of Hes7-null embryos, as in the wild type (Fig. 4D). Therefore, Hes7 mutation did not significantly affect expression of the PSM genes, except for its own promoter activity.

To determine the A–P polarity of formed somites, we next examined the anterior-half- or posterior-half-specific markers. The homeobox gene Uncx4.1, which controls sclerotome development (Mansouri et al. 2000), was expressed in the posterior half of each somite of the wild type (Fig. 4E,F). In contrast, in Hes7-null mice, the expression was severely disorganized (Fig. 4E,F). Interestingly, the disruption of Uncx4.1 expression was milder in the rostral part (Fig. 4E,F, arrows) than in the caudal part (Fig. 4E,F, brackets), which contained newly formed somites. At E8.5, the first four Uncx4.1+ somites were irregularly separated (Fig. 4E, arrows), whereas the posterior somites were fused in Hes7-null embryos (Fig. 4E, bracket). In contrast, at E9.5, the first 10 somites were separated and the Uncx4.1 expression was restricted to the posterior half of each somite in Hes7-null embryos (Fig. 4F, arrows), whereas the caudal somites were fused (Fig. 4F, bracket). Therefore, it is likely that the initial segmentation and A–P patterning defects of somites are partially corrected at later stages in Hes7-null embryos. This correction could be involved in sorting of the anterior-half and posterior-half somite cells because they are known to have different adhesive properties (Stern and Keynes 1987). Expression of another posterior marker, Ephrin-B2, became very weak and almost undetectable in the somites of Hes7-null mice, whereas it was observed in the PSM (Fig. 4G). The anterior somite marker EphA4 was expressed in two domains in wild-type embryos—the anterior half of somite I (Fig. 4H, arrowhead) and the anterior region of the PSM (Fig. 4H, arrow). In Hes7-null embryos, EphA4 expression in somite I became almost undetectable, whereas the expression in the PSM was not significantly affected (Fig. 4H). These results indicate that the A–P polarity of somites was severely disorganized in Hes7-null mice.

Because Hes7 is a putative downstream effector of the Notch pathway, we next analyzed the expression of Notch receptors and ligands. In the wild type, Notch1 was expressed in the PSM with a sharp anterior border between somites I and –I, whereas in the Hes7-mutant embryo, the expression in the PSM was observed but the anterior border was diffuse (Fig. 5A). Notch2 was expressed in the anterior half of the newly formed somites and the whole region of the next presumptive somite (somite –II of the wild type, whereas in Hes7-null embryos, only a broader expression domain was observed in the anterior PSM (Fig. 5B). The Notch ligand genes Dll1 and Dll3 were expressed in the PSM with a sharp anterior border in the wild-type embryo (Fig. 5C,D). These genes were expressed in the mutant PSM, although the anterior border was more diffuse and Dll1 expression in the posterior half of each somite became almost undetectable (Fig. 5C,D). These results indicate that, although expression of the receptors and ligands of Notch signaling became diffuse at the anterior border of the PSM and undetectable in somites, the overall expression level in the PSM was not affected by loss of Hes7.

**Disruption of oscillating gene expression in Hes7-mutant mice**

We next examined expression of the oscillator genes Hes1 and Hey2 (Jouve et al. 2000, Leimeister et al. 2000). In the wild type, both Hes1 and Hey2 were expressed in the PSM and the posterior half of somites (Fig. 5E,F). In the Hes7-null embryo, although Hes1 expression remained in somite –I (Fig. 5E, bracket), it became undetectable in the posterior PSM and somites (Fig. 5E). In addition, Hey2 expression was also undetectable in the PSM of Hes7-null embryos (Fig. 5F). Therefore, Hes7 is essential for Hes1 and Hey2 expression in the PSM. We next examined another oscillator gene, Lfng. In the wild-type and heterozygous embryos, Lfng displayed various expression patterns categorized into three stages (Fig. 5G). In contrast, we did not observe such variability of Lfng expression in Hes7-null embryos (Fig. 5H). Strikingly, all Hes7-null embryos displayed the same expression pattern \( n = 8 \) at E8.5 and \( n = 9 \) at E9.5. The expression was observed continuously throughout the PSM with the highest in the anterior PSM, probably at somite –II (Fig. 5H). In addition, the same abnormal expression pattern was observed at both E8.5 and E9.5 (Fig. 5H), indicating that the defect of Lfng expression is not progressing but rather constant. This expression pattern of Lfng was never observed at any stages in the wild-type and heterozygous embryos (Fig. 5G). These results strongly indicate that the oscillating expression of Hes1,

**Figure 4.** Defects of somitogenesis in Hes7-null embryos. (A) Whole-mount lacZ staining. In a Hes7+/- embryo, weak staining is observed in the PSM and several caudal somites. In contrast, in a Hes7-/- embryo, much stronger staining is observed in the PSM and irregular somites. A higher magnification is shown in the lower panels. (B–H) Expression of Paraxis (B), pMesogenin (C), MesP2 (D), Uncx4.1 (E,F), Ephrin-B2 (G), and EphA4 (H) was examined by in situ hybridization. (E,F) The disorder of Uncx4.1 expression is more severe in the caudal parts [brackets] than in the rostral parts [arrows]. In E, rostral is to the left. (G) Ephrin-B2 expression disappears in somites but remains in the PSM of Hes7-null embryos. (H) In the wild type, EphA4 is expressed in the anterior half of somite I (arrowhead) and the PSM (arrow). In Hes7-null embryos, the expression in somite I disappears.

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but undetectable in other regions (n type, Hes7 of somites (arrowheads). In the embryos (E8.5, 9; stage III, 10 out of 25 embryos) and (16; stage III, 12 out of 37 embryos) and displays variable expression patterns at E8.5 (stage I, 9; stage II, 16; stage III, 12 out of 37 embryos) and E9.5 (stage I, 6; stage II, 9; stage III, 10 out of 25 embryos). (H) In contrast, all Hes7-null embryos (E8.5, n = 8; E9.5, n = 9) exhibit the same expression pattern—Lfg is expressed continuously throughout the mutant PSM.

Hey2, and Lfg in the PSM was all disrupted in Hes7-null mice. Here, we showed that mutation for a new member of the Hes family, Hes7, leads to severe defects of somite segmentation. Strikingly, in Hes7-null embryos, Lfg expression was expressed continuously throughout the PSM, and therefore the cyclic expression of Lfg was disrupted. Because Hes7 is a transcriptional repressor (Bessho et al. 2001), it may down-regulate Lfg expression and generate a Lfg-negative region in the PSM during oscillation. Our data do not, however, exclude the possibility that Lfg expression still oscillates in individual cells in Hes7-null embryos. Interestingly, the phenotypes of Hes7-deficient mice are very similar to those of Lfg-null mice (Evard et al. 1998). Therefore, both null mutation and continuous expression of Lfg result in somite defects, suggesting that the oscillating expression is essential for coordinated somite segmentation.

Although Hes7 seems to repress Lfg and its own expression, both Hes7 and Lfg mRNAs are generated in the same phase during oscillation in the PSM, suggesting that Hes7 protein is unlikely to function in Lfg/Hes7 mRNA* domains. Previous analysis of the immediate-early gene c-fos suggests that the peak of the protein level is ~30 min later than that of the mRNA level (Müller et al. 1984). Therefore, the synthesis of Hes7 protein is likely to be delayed compared with the production of Hes7 mRNA. We speculate that the Hes7 protein may function in the regions just caudal to the peak of Lfg and Hes7 mRNA* domain, although further studies are required to determine the position of the peak of Hes7 protein level.

Materials and methods

Generation of Hes7 mutant mice

Targeting vector was constructed by replacing most of the Hes7-coding region to IRES-LacZ and PGK-neo (Fig. 2A). ES cell lines with Hes7 mutation were identified from Southern blot analysis using 5' and 3' probes (Fig. 2A B). Chimeric mice were generated and bred with ICR mice, as described previously (Tomita et al. 2000).

Explant culture of mouse embryos

Whole-mount in situ hybridization of mouse embryos was performed as described previously (Bessho et al. 2001). Notch1, Notch2, andDll1 cDNAs were kindly provided by Dr. Gerry Weinmaster. Other probes were obtained by RT–PCR and the following regions were used: Dll3, nucleotide residues 612–1341; Lfg, 17–1382; Mesp2, 1–1697; Ucnex4.1, 14–1690; Paraxis, 106–837; pMesogenin, 2–547; EphA4, 1550–2960; EphrinB2, 1–2143; Hes1, 178–1213; and Hey2, 135–1020. To assess the expression of the LacZ gene, embryos were fixed in 0.2% glutaraldehyde and then stained in 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl2, and 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

Skeletal preparation

Cartilages and bones of newborn mice were stained with alizarin red after fixation in 5% ethanol as described by Inouye (1976).

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