RESEARCH COMMUNICATION

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 derivatives such as vertebrae and ribs are severely disorganized. Although expression of Notch and its ligands is not affected significantly, the oscillator and Notch modulator *lunatic fringe* is expressed continuously throughout the mutant PSM. These results indicate that *Hes7* controls the cyclic expression of *lunatic fringe* and is essential for coordinated somite segmentation.

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Somites, the segmental units for the metameric structures such as skeletal muscles, vertebrae, and ribs are sequentially generated in a head-to-tail order by segmentation of the mesenchymal tissue called the presomitic mesoderm (PSM). The segmentation occurs periodically at the anterior end of the PSM, and this periodic segmentation has been suggested to be regulated by a molecular clock (Cooke 1998; Dale and Pourquié 2000). The first evidence for the molecular clock for somite segmentation was provided by a report about the chick basic helix–loop–helix (bHLH) gene *chairy1* (Palmeirim et al. 1997). *chairy1* expression initiates as a broad band in the posterior PSM, moves anteriorly, and reaches the anterior end of the PSM as a narrow band, which corresponds to the posterior part of a newly formed somite (Palmeirim et al. 1997). This sequence of *chairy1* expression is repeated, and each cycle is correlated with formation of one somite. Similar dynamic expression is also observed for other *chairy1*-related bHLH genes, such as zebrafish *Her1* and mouse *Hes1* (Holley et al. 2000; Jouve et al. 2000; Sawada et al. 2000). Interestingly, the dynamic *chairy1* expression is not the result of cell movement but attributable to wave-like propagation through the PSM

cells (Palmeirim et al. 1997). For the wave-like propagation, *chairy1* expression should oscillate synchronously in the PSM, but the exact mechanism for the synchronous oscillation remains to be determined.

Recent studies suggest that Notch signaling regulates the synchronously oscillating gene expression in the PSM and coordinated segmentation of somites. In zebrafish, mutation for the Notch pathway is likely to desynchronize the oscillating gene expression (a random mixture of PSM cells that express different levels of gene expression) and this desynchronization may lead to a loss of the coordinated somite segmentation (Holley et al. 2000; Jiang et al. 2000). Similarly, in mice, Notch signaling is essential for oscillating gene expression (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; Hrabe de Angelis et al. 1997; Wong et al. 1997; Evrard et al. 1998; Kusumi et al. 1998; Zhang and Gridley 1998; del Barco Barrantes et al. 1999; Donoviel et al. 1999; 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 (E)9.5

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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 the 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 *Uncx4.1* 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.

mouse embryos were cut into halves along the midline and the two halves were subjected 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; during stage II, the expression moves anteriorly and becomes narrower; and during stage III, the expression is present in somite –II (somite –I is the next somite to form and somite –II is the one to form after somite –I). During stage I, a broad *Hes7* expression domain appeared 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* expres-

sion 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

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. Diphtheria 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).

Figure 3. Segmentation defects in *Hes7*-null mice. (A–F) Neonates of heterozygous (A,C,E) and homozygous (B,D,F) mutants were stained for bone (red) and cartilage (blue). (A,B) Dorsal view of the whole neonates; (C,D) lateral view of vertebrae and; (E,F) dissected ribs and sternums. (G,H) HE staining of sagittal sections of *Hes7*(+/-) (G) and *Hes7*(-/-) (H) neonates. In *Hes7*(-/-) embryos, vertebral bodies and neural arches (arrows) are fused, and the patterning of vertebral bodies and intervertebral disks is defective. (ID) Intervertebral disk; (NA) neural arch; (SC) spinal cord; (VB) vertebral body. (I,J) Parasagittal sections of E9.5 embryos. Compared with *Hes7*(+/-) (I), somites are irregularly arranged in *Hes7*(-/-) embryos (J). Incomplete and almost complete fusion of epithelial somites are indicated by arrow and arrowhead, respectively. Bar, 1 mm (G,H); 100 μm (I,J).

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-

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.

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 ante-

rior-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 somite and the whole region of the next presumptive somite (somite -I) 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 5. Whole-mount in situ hybridization for Notch signaling molecules. (A) In the *Hes7*-null embryo, the expression is not significantly affected in the PSM, but the anterior border becomes diffuse. (B) In the wild type, *Notch2* is expressed in somites I (arrow) and -I (arrowhead). In the *Hes7*-null embryo, only a broader expression is observed in the anterior PSM. (C) In the wild type, *Dll1* is expressed in the PSM and the posterior half of each somite. In the *Hes7*-null embryo, it is expressed in the PSM, but the anterior border is diffuse and the somite expression is undetectable. (D) *Dll3* expression in the PSM is not significantly affected but the anterior border is diffuse in *Hes7*-null embryos. (E) In the wild type, *Hes1* is expressed in the PSM (brackets) and the posterior half of somites (arrowheads). In the *Hes7*-null embryo, the expression is observed in somite -I (bracket) but undetectable in other regions ($n = 5$). (F) In the wild type, *Hey2* is expressed in the PSM (arrow) and the posterior half of somites (arrowheads). In the *Hes7*-null embryo, the expression is almost undetectable ($n = 4$). (G) In the wild type, *Lfng* displays various 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—*Lfng* is expressed continuously throughout the mutant PSM.

Hey2, and *Lfng* in the PSM was all disrupted in *Hes7*-null mice.

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

1998). Therefore, both null mutation and continuous expression of *Lfng* result in somite defects, suggesting that the oscillating expression is essential for coordinated somite segmentation.

Although *Hes7* seems to repress *Lfng* and its own expression, both *Hes7* and *Lfng* mRNAs are generated in the same phase during oscillation in the PSM, suggesting that *Hes7* protein is unlikely to function in *Lfng*/*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 *Lfng* 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 by 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

The caudal part of E9.5 mouse embryo was bisected along the midline in fetal bovine serum. The explants were cultured on polycarbonate filters (Millipore) in DMEM (GIBCO) supplemented with 20% fetal bovine serum, as described previously (Palmeirim et al. 1997).

In situ hybridization and lacZ staining

Whole-mount in situ hybridization of mouse embryos was performed as described previously (Bessho et al. 2001). *Notch1*, *Notch2*, and *Dll1* 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; *Lfng*, 17–1382; *Mesp2*, 1–1697; *Uncx4.1*, -14–1680; *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 alcian blue and alizarin red after fixation in 95% ethanol as described by Inouye (1976).

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