Hes7 3’UTR is required for somite segmentation function

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A set of genes in the posterior end of developing mouse embryos shows oscillatory expression, thereby regulating periodic somite segmentation. Although the mechanism for generating oscillation has extensively been clarified, what regulates the oscillation period is still unclear. We attempted to elongate the oscillation period by increasing the time to transcribe Hes7 in this research. We generated knock-in mice, in which a large intron was inserted into Hes7 3’UTR. The exogenous intron was unexpectedly not properly spliced out and the transcripts were prematurely terminated. Consequently, Hes7 mRNA lost its 3’UTR, thereby reducing the amount of Hes7 protein. Oscillation was damped in the knock-in embryos and periodic somite segmentation does not occur properly. Thus, we demonstrated that Hes7 3’UTR is essential to accumulate adequate amounts of Hes7 protein for the somite segmentation clock that orchestrates periodic somite formation.

Somitogenesis is the most prominent cyclic event in vertebrate development. Somites are sequentially formed in the anterior-posterior direction. A pair of somites buds off from the anterior part of the pre-somatic mesoderm (PSM), i.e., the unsegmented mesenchymal tissue in the posterior embryo of vertebrates, in a rhythmic fashion. Due to this temporal periodicity, the array of somites is formed as a spatial periodic pattern. Somites give rise to vertebrae, ribs, skeletal muscles, and skin, and thus, somites are the earliest segmental units of the vertebrae body1,2. The period of somite formation is characteristic of the species, e.g., 2 h in mice, 90 min in chicks, and 20–30 min in zebrafish3. Because the timing for the beginning and end of somite formation is strictly determined during development, the number of somites and the number of resulting vertebrae may be dependent on the period of somitogenesis.

The periodicity of somitogenesis is instructed by the synchronous oscillation of gene expression in PSM, which is termed the somite segmentation clock1. The expression of a set of genes oscillates in a 2-h cycle in mouse PSM, where expression demonstrates cyclic wave-like propagation from the posterior end of the embryo to the anterior PSM. A pair of somites is generated during each cycle1. The oscillating genes in mouse include components of Notch signaling, fibroblast growth factor (FGF) signaling, and Wnt signaling4. One of these genes is an effector gene of Notch signaling, Hes7, which encodes a basic helix-loop-helix type transcription factor5. Hes7, as a transcriptional repressor, binds to its own promoter, thereby inhibiting its own transcription6,7. Thus, Hes7 forms a negative feedback loop, which is the major mechanism for oscillatory gene expression. Hes7 also inhibits the transcription of several target genes; therefore, the expression of these genes oscillates in a synchronized manner8. These target genes of Hes7 include lunatic fringe (Lfng), which encodes a glycosyl-transferase that modulates the activity of Notch signaling9. The activity of Notch signaling also oscillates in the PSM due to the oscillation of Lfng, and this dynamic Notch activity contributes to the mechanism for somite segmentation9.

Mathematical models have been proposed based on this mechanism for the transcriptional regulation that generates oscillatory gene expression in the somite segmentation clock10-13. Of these, a mathematical model that directed the negative feedback loop of a transcription factor with its time delay successfully reproduced oscillatory gene expression12,13. The predicted period of oscillation in this model mainly depended on the time delay of the negative feedback loop. The time delay included the time taken to transcribe and process mRNAs, synthesize and modulate proteins, and traffick mRNAs and proteins. One acceptable way to test and verify the model, in which the feedback loop of Hes7 plays a central role, is to manipulate parameters in the model and to detect its resulting
phenotype in somitogenesis. For instance, increasing the time for mRNA transcription should increase the time delay, thereby extending the period of gene oscillation and somite segmentation in the model.

We tried to increase the period of gene oscillation and somite segmentation in mouse somitogenesis in this research by increasing the time taken to transcribe Hes7. We inserted a large intron derived from human dystrophin into the 3’UTR region of mouse Hes7 in ES cells, and generated knock-in mice. Previous researches have attempted to increase the time delay in the somite segmentation clock by using similar strategies. Staub et al. inserted large intron sequences derived from human dystrophin into the third intron of mouse Hes7, and Hanisch et al. introduced a her1 transgene that they inserted a large sequence into the second intron into a her1/7 mutant zebrafish. The large introns were unexpectedly not correctly spliced out in both studies, and both groups failed to increase the time delay in the somite segmentation clock. The exogenous intron was coincidentally not properly spliced out in this study, and thus we also failed to increase the time taken to transcribe Hes7. The transcripts of the knock-in Hes7 allele were prematurely terminated within the intron sequence in the mouse PSM cells. Consequently, Hes7 mRNA lost its 3’UTR. Oscillatory gene expression in PSM was lost in the embryos of the knock-in mice and no adequate periodic somite segmentation occurred. Accordingly, the metameric pattern of somites was severely affected, thereby severely disrupting the axial skeletons. This phenotype is similar to that in Hes7 null mutants. In addition, the amount of Hes7 protein was severely reduced in the PSM of the knock-in mice. Thus, Hes7 3’UTR was essential to generate sufficient amounts of Hes7 protein. Altogether, our results demonstrated that Hes7 3’UTR is required for the somite segmentation function.

Results

Generation of Knock-in mice. Hes7 protein inhibits its own transcription to form a negative feedback loop, which is supposed to be the core mechanism for gene oscillation. Previous research using mathematical modeling has proposed that the time delay from Hes7 transcription to the accumulation of Hes7 protein is the critical factor in determining the oscillation period. We tried to lengthen the time delay by increasing the size of the Hes7 gene to verify the prediction, and observed whether the oscillation period was extended. We inserted 5, 10, and 20 kb exogenous intron sequences derived from human dystrophin into embryonic stem (ES) cells at the 3’UTR of the Hes7 gene by homologous recombination (Fig. 1a: see Methods). The recombinants were confirmed by Southern blotting and polymerase chain reaction (PCR) analyses (Fig. 1b,c, data not shown). The transcription elongation rate of RNA polymerase II was previously estimated by several groups, and they worked out different values from 1.1–4.8 kb/min. If we had taken 1.1 kb/min as the velocity of the RNA polymerase on the Hes7 allele on the one hand, we would have expected that 5, 10, and 20 kb exogenous intron sequences would respectively increase the time delay by 4.5, 9, and 18 min. If we had assumed it to be 4.8 kb/min on the other hand, we would have expected that 5, 10, and 20 kb exogenous intron sequences would respectively increase the time delay by 1, 2.1, and 4.2 min. We carried out a simulation according to our mathematical model to estimate the expected oscillation period with these alterations (Supplementary note). Our model predicted that the increment of time delay led to sustained oscillations, and that 2.1–9 min additional time delay brought by the 10 kb exogenous intron sequence increased the oscillation period by 4.9–20.2 min. The exogenous intron sequences were expected to be spliced out, and consequently only 89-base insertion derived from loxP and the human dystrophin sequence should remain in the 3’UTR of the mature transcripts from the mutant Hes7 allele. The resulting Hes7 protein should be exactly the same as that of the wild type.

Mutant mice were generated from the recombinant ES cells. We found that Hes7/10k mice, Hes7/10k mice, and Hes7/20k mice had short trunks and tails (Fig. 1d, data not shown). The short trunks and tails were unexpected phenotype because our mathematical simulation predicted that increasing the time delay would lead to the oscillation period being altered, but that sustained oscillation would still be maintained.

Homologous mutant mice with defects in somite formation and consequent segmental defects. Because the phenotypes of short trunks and tails resembled those of Hes7 null mutant mice (Hes7−/−), which have anomalous axial skeletons, we examined the axial patterning in Hes7/10k, Hes7/10k, and Hes7/20k neonates. The vertebral and ribs of neonates were stained with alizarin red and alcan blue. All Hes7/10k, Hes7/10k, and Hes7/20k neonates had segmentation defects in their vertebral and ribs, and their phenotypes were similar (Fig. 2c–e). Segmental defects were...
observed throughout the vertebrae and ribs of all Hes7-/-, Hes7 5/10-/-, and Hes7 10k/10k neonates, which were the same as the skeletal phenotypes in Hes7-/- neonates. However, the axial skeletons of Hes7 null neonates shrank much more than those of Hes7-/-, Hes7 5/10-/-, and Hes7 10k/10k neonates (Fig. 2b–e). We expected that the Hes7 5/10-/-, Hes7 10k/10k, and Hes7 20k/20k mice would have longer oscillation periods from our mathematical simulation, which would lead to fewer somites/vertebrae. In addition, because the expected oscillation period in Hes7 20k/20k mice was longer than that in Hes7 5/10-/- or Hes7 10k/10k mice, the Hes7 20k/20k mice were expected to have fewer vertebrae than in Hes7 10k/10k or Hes7 7k/7k mice. However, there were no differences in the numbers of vertebrae in Hes7 5/10-/-, Hes7 10k/10k, and Hes7 7k/7k neonates. We decided to examine Hes7 10k/10k mice in the analyses that followed because the phenotypes of all Hes7 5/10-/-, Hes7 20k/20k, and Hes7 5/20k/20k neonates were similar.

Because the segmental patterns of vertebrae and ribs were derived from the metameric pattern of somites in the embryonic stage, we next examined somite patterning in Hes7 5/10-/- embryos. A homeobox gene, Uncx4.1, was exclusively expressed in the posterior half of each somite, thereby displaying an ordered ladder pattern in the wild-type embryos (Fig. 3a). The expression of Uncx4.1 was severely disrupted in the absence of Hes7 (Fig. 3c), as has previously been reported. The signals of Uncx4.1 were fused in Hes7 5/10-/- embryos and the ladder pattern was disrupted (Fig. 3b). Another somite marker, myogenin, which was expressed in the myotome in each somite, also exhibited an ordered ladder pattern in wild-type embryos (Fig. 3d). However, the signals of myogenin were fused in some parts in Hes7 5/10-/- embryos (Fig. 3e) as same as in Hes7-/- embryos (Fig. 3f). These results indicate that somites are not sufficiently segmented in Hes7 5/10-/- embryos, and this anomaly in somites probably leads to segmental defects in neonates.

Amount of Hes7 protein is reduced in Hes7 7k/10k embryos. We detected Hes7 mRNA with a probe derived from the Hes7 coding sequence in wild-type and Hes7 5/10-/- embryos at E 10.5 to check the expression patterns of cyclic genes in Hes7 10k/10k embryos. The expression of Hes7 revealed wave patterns in the PSM of wild-type embryo, as has previously been reported. In contrast, the expression of Hes7 mRNA spread uniformly in PSM of Hes7 10k/10k embryos, and did not exhibit wave patterns (Fig. 4b).

We speculated that the amount of Hes7 mRNA and/or Hes7 protein in Hes7 10k/10k embryos would be insufficient to maintain sustained oscillation because the transcription of Hes7 is activated through the PSM cells in Hes7 null embryos. Thus, we assessed the amount of Hes7 mRNA and Hes7 protein derived from the Hes7 10k allele in Hes7 10k/10k embryos. We found from quantitative PCR that the amount of Hes7 mRNA was reduced by 30% in the PSM of Hes7 10k/10k embryos in comparison with wild-type embryos. We then carried out immunohistochemistry with an anti-Hes7 antibody. The Hes7 protein in wild-type embryos revealed a wave pattern in the PSM (Fig. 4c). In contrast, we could hardly detect Hes7 protein in the PSM of Hes7 10k/10k embryos (Fig. 4d). These results suggest that the amount of Hes7 protein derived from the Hes7 10k allele was grossly reduced in PSM, and that Hes7 protein was not effectively produced in Hes7 10k/10k embryos. This is consistent with the phenotypes of Hes7 10k/10k embryos, which are similar to Hes7 null mutants.

Transcriptional activities of cyclic genes up-regulated by reduced amount of Hes7 protein in Hes7 10k/10k embryos. Because Hes7 cyclically represses its own transcription and transcription of cyclic genes including Lfg, thereby generating synchronized gene oscillation in PSM, we assumed the transcriptional activities of Hes7 and Lfg would be enhanced in knock-in mice. Thus, we first examined the transcriptional activity of the Hes7 10k allele. The transcriptional activity can be evaluated by detecting the intron
fragments in transcripts because the intron sequences in transcripts are spliced out immediately after transcription and the intron fragments are degraded immediately after splicing. We performed in situ hybridization with a probe derived from the first intron of Hes7 to detect regions active in the transcription of Hes7. The regions active in the transcription of Hes7 displayed various patterns in the PSM of wild-type embryos, which is consistent with the expression pattern of Hes7 mRNA (Fig. 4e). However, the entire PSM of Hes7^10k/10k embryos was active in the transcription of the Hes7^10k allele (Fig. 4f). We carried out a quantitative reverse transcription-polymerase chain reaction (RT-PCR) to quantitatively measure transcriptional activity. We detected the sequence of the first intron of Hes7 in the transcripts, and found that the transcriptional activity of the Hes7^10k allele in the PSM of Hes7^10k/10k embryos was substantially up-regulated in comparison with that of the Hes7 allele in the PSM of Hes7^10k/10k embryos. These results suggest that the transcriptional activity of the Hes7^10k allele is up-regulated in the PSM of Hes7^10k/10k embryos, probably because of reduced amount of Hes7 protein.

Next we examined the expression of Lfng, which is one of the target genes of Hes7. As expected, Lfng was uniformly expressed throughout the whole PSM of Hes7^10k/10k embryos (Fig. 4h). We carried out quantitative RT-PCR analyses to quantitatively evaluate Lfng expression. We found that the transcriptional activity of Lfng was clearly up-regulated in the PSM of Hes7^10k/10k embryos in comparison with that in the PSM of wild-type embryos (Fig. 5d). The amount of Lfng mRNA was also slightly increased in the PSM of Hes7^10k/10k embryos (Fig. 5e). These results suggest that the reduced amount of Hes7 enhanced the expression of Lfng, and that sufficient amounts of Hes7 are essential for oscillatory gene expression and somite segmentation.

**Hes7^3′UTR is essential for somite segmentation clock.** The 10 kb intron derived from human dystrophin in the Hes7^10k allele was inserted immediately downstream of the stop codon of Hes7 (Fig. 6a). We carried out RT-PCR with RNAs extracted from the PSMs of wild-type and Hes7^10k/10k embryos at E 10.5 to check whether this exogenous intron was spliced out to form appropriate mRNAs. We designed a forward primer, F1, in the 4th exon, a reverse primer, R1, in 3′ UTR, and two reverse primers, R2 and R3, in the intron derived from dystrophin (Fig. 6a–c, 7a). We detected Hes7 mRNA in wild-type embryos, whereas we failed to obtain a PCR product in Hes7^10k/10k embryos with the F1 and R1 primers (Fig. 7b). Although a PCR product was detected in Hes7^10k/10k PSMs by combining the F1 and R2 primers, no products were detected by combining the F1 and R3 primers (Fig. 7b). These results suggest that the human dystrophin intron inserted into Hes7^3′UTR was not spliced out in Hes7^10k/10k PSMs, and that the transcripts of the Hes7^10k allele terminated between the positions of the R2 and R3 primers.

We carried out in situ hybridization with a probe derived from Hes7^3′UTR to further examine the structure of Hes7 mRNA in Hes7^10k/10k embryos. We detected Hes7 mRNA in wild-type embryos, whereas we failed to detect Hes7 mRNA in Hes7^10k/10k embryos (Fig. 8a,b). In contrast, we uniformly detected mRNAs in the PSM of Hes7^10k/10k embryos by using a probe derived from the 5′ end of the dystrophin intron (Fig. 8d), and the pattern was similar to the pattern obtained with the probe derived from the coding of Hes7 (Fig. 4b). No signal was detected with the dystrophin-intron probe in wild-type embryos because this intron was adopted by the human dystrophin (Fig. 8c). These results support our interpretation that the exogenous intron was not spliced out in the Hes7^10k transcripts, which were prematurely terminated between the positions of the R2 and R3 primers.

We carried out 3′-rapid amplification of cDNA ends (3′-RACE) to identify the positions of the 3′ end of the transcripts of Hes7^10k. We detected several PCR products, and two of them were derived from the Hes7^9 allele (Fig. 8e). One was terminated at a site 2.4 kb downstream of the stop codon. The other was terminated at a site 3.3 kb downstream of the stop codon. Because the 3′ ends of both products were located between the positions of the R2 and R3 primers, this was consistent with the results obtained from RT-PCR analysis (Fig. 6a,b). We found polyadenylation site-like sequences (AATAAA) just upstream of the 3′ ends of both transcripts (Fig. 6c,8e). Thus, these results suggest that sequences similar to the polyadenylation site in the exogenous human dystrophin intron were misidentified, thereby leading to premature termination and adding polyadenylation tails to the Hes7^10k transcripts. Hes7^3′UTR was essential to generate sufficient amounts of Hes7 protein to maintain the somite segmentation clock, taking all results into account.

**Discussion**

We manipulated the mouse Hes7 gene in ES cells, with which we generated lines of knock-in mice. The proper 3′ UTR of Hes7 mRNA was replaced by an exogenous sequence derived from the human dystrophin intron by modifying the Hes7 gene. This alteration of 3′ UTR reduced the amount of Hes7 mRNA and Hes7 protein, leading to gene oscillation in PSM being damped and to the failure of periodic somite formation. We therefore concluded that Hes7 played the major role in generating gene oscillation in PSM, where Hes7^3′UTR was essential to generate sufficient amounts of Hes7 protein.

We originally attempted to increase the time for mRNA transcription by increasing the gene size of Hes7, thereby expanding the oscillation period. We expected that the extended oscillation period

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**Figure 4** | Expression patterns of Hes7 and Lfng, and distribution of Hes7 protein in PSM. (a,b) Distribution of Hes7 mRNA in PSM of wild-type (n = 6) (a) and Hes7^10k/10k (n = 3) (b) embryos at E 10.5. (c,d) Distribution of Hes7 protein in PSM of wild-type (n = 15) (c) and Hes7^10k/10k (n = 17) (d) embryos at E 10.5. Whole-mount immunostaining was carried out with anti-Hes7 antibody. Lateral views were shown, and white dotted lines represent the contours of PSM. Left panels show the signal for Hes7 immunostaining and right panels show the merged views with the nuclei stained with Hoechst33258 (e,f). Regions active in the transcription of Hes7 detected using Hes7 intron probe in PSM of wild-type (n = 5) (e) and Hes7^10k/10k (n = 4) (f) embryos at E 10.5. (g,h) Distribution of Lfng mRNA in PSM of wild-type (n = 12) (g) and Hes7^10k/10k (n = 8) (h) embryos at E 10.5. Scale bars: 100 μm.

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would decrease the number of somites and vertebrae and/or increase the size of somites. However, the knock-in mice did not exhibit the expected phenotypes because the inserted introns decreased the amount of \(Hes7\) mRNA and \(Hes7\) protein in PSM, thereby leading to damped oscillation.

Stauber et al. recently independently attempted to elongate time delay in the segmentation clock\(^{14}\). They generated knock-in mice, where the size of the third intron of \(Hes7\) was increased. However, this modification caused incorrect splicing, thereby drastically reducing the activity of \(Hes7\). Consequently, the homozygous knock-in mutant embryos demonstrated similar phenotypes in somites and axial skeletons as the \(Hes7\) knock out embryos. Thus, they failed to increase the time delay in knock-in mice. Hanisch et al., tried to rescue the mutants with the transgene because the modified intron was not correctly spliced out. Nevertheless, they measured the velocity of RNA polymerase II on the allele by using the transgene, and demonstrated that it was 4.8 kb/min, which was much faster than they expected. According to the results, they discussed that the contribution of time taken for transcription to the time delay in the segmentation clock was much shorter than that of time taken for other steps including the splicing and export of mRNA. Hoyle and Ish-Horowicz measured the time to splice and export the mRNA of cyclic genes in mouse, chick and zebrafish PSM\(^{24}\). Although they tried to estimate the time to transcribe the genes in vivo, they failed because the velocity of transcription was too fast. The time delay in the feedback loop of \(Hes7\) contains the time for several steps including transcription, mRNA processing, protein translation, protein modification, and mRNA and protein trafficking. These reports concluded that the contribution of time in these steps taken for transcription to the time delay was much smaller than that of time taken for other steps\(^{15,24}\). However, it is quantitatively difficult to alter the time for these steps because their kinetics is not well understood. Thus, although three...
groups, including us, failed practically to elongate the time to transcribe \(Hes7\)14,15, manipulating the gene size remains a possibility to increase the oscillation period. Conversely, Takashima et al. tried to shorten time delay by removing the introns from the \(Hes7\) gene25. They estimated that introns led to about a 19-min delay in the \(Hes7\) gene. The \(Hes7\) gene has a total length of about 2.8 kb6, and RNA polymerase was speculated to have a velocity of 1.1–4.8 kb/min15–20. Thus, the time to transcribe introns should be less than 2 min. Most of the time delay created by introns is probably due to the process of splicing. They found that gene oscillation in mutant mice was damped, and that segmentation was severely affected. Thus, they concluded that the time delay derived

**Figure 6 | Structure of \(Hes7^{10k}\) allele and its transcript.** (a) Structure of \(Hes7^{10k}\) allele. The sequence from just upstream of the transcription initiation site of \(Hes7\) to just downstream of the polyadenylation site of \(Hes7\) is shown. Upstream and downstream sequences of the \(Hes7\) gene, and the human \(dystrophin\) intron sequence are indicated by the small letters. (b) The structure of the anticipated transcripts of the \(Hes7^{10k}\) allele. (c) The structure of the transcripts of the \(Hes7^{10k}\) allele that were detected in PSM. The positions of the primers (F1, R1, R2, and R3) are indicated by the red arrows. The \(Hes7\) 3'UTR and 5'UTR are indicated by the open boxes and the \(Hes7\) coding region is indicated by the closed box. The sequences corresponding to primers, mutants of \(loxp\), and human \(dystrophin\) exon are indicated by the red, green, and blue letters. The polyadenylation site and polyadenylation site-like sequences are indicated by boxes (AATAAA). The initiation codon (Met) and the termination codon (stop) are underlined.
from introns was essential for gene oscillation in the segmentation clock. The same group also found that deleting two introns from the human dystrophin gene is indicated by the blue lines. The red arrows indicate the position of the primers for RT-PCR analyses. (b) RT-PCR analyses of the transcript of the Hes7 allele. Total RNA was extracted from the PSMs of wild-type or Hes7/10k/10k embryos, and was analyzed by RT-PCR with the combinations of indicated primers (panels on left). Genomic DNA was extracted from wild-type or Hes7/10k/10k mice. PCR analyses were carried out with combinations of indicated primers (panels on right). Gapdh mRNA was detected as a positive control. The combination of F1 and R1 primers failed to detect the 10 kb band with genome DNA or the mRNA of Hes7/10k/10k mice.

Figure 7 | Characterization of transcript of Hes7 allele. (a) Structures of Hes7 gene and Hes7 mRNA are provided in upper panel. The bottom panel indicates the structure of the Hes7 allele and its transcript. The intron sequence derived from the human dystrophin gene is indicated by the blue lines. The red arrows indicate the position of the primers for RT-PCR analyses. (b) RT-PCR analyses of the transcript of the Hes7 allele. Total RNA was extracted from the PSMs of wild-type or Hes7/10k/10k embryos, and was analyzed by RT-PCR with the combinations of indicated primers (panels on left). Genomic DNA was extracted from wild-type or Hes7/10k/10k mice. PCR analyses were carried out with combinations of indicated primers (panels on right). Gapdh mRNA was detected as a positive control. The combination of F1 and R1 primers failed to detect the 10 kb band with genome DNA or the mRNA of Hes7/10k/10k mice.

The combination of F1 and R1 primers failed to detect the 10 kb band with genome DNA or the mRNA of Hes7/10k/10k mice.

the shorter intron (5 kb) also failed to be spliced out. Using other introns instead of those derived from human dystrophin may solve the problem.

Nonsense-mediated mRNA decay (NMD) is a well-studied mechanism that selectively eliminates abnormal mRNAs. An intron located downstream of the stop codon is detected in the NMD mechanism, thereby the transcript is rapidly degraded. However, if the distance between the stop codon and the intron is less than 50–55 bp, the transcripts are not degraded by NMD. Therefore, we placed the exogenous intron just downstream of the stop codon of Hes7 to avoid NMD. The transcripts were not subjected to NMD because the exogenous intron in the mutant Hes7 transcript was not spliced out in the Hes7/10k/10k embryo. Thus, the small reduction of Hes7 mRNA in the Hes7/10k/10k embryo is not likely due to NMD.
10 bp of the 5' UTR of Hes7 mRNA was reduced, and this was probably responsible for the phenotype in the mutant embryos. Thus, we concluded that Hes7 3' UTR is essential for maintaining the amount of Hes7 protein. We recently demonstrated that the 3'UTRs of Hes7 and Lfng were responsible for the differential distribution patterns of their mRNAs in PSM. This is consistent with our findings in this report, which has accumulated evidence on the importance of 3' UTR in gene oscillation. We demonstrated in this research that the amount of Hes7 mRNA was reduced by 30% in the PSM of Hes7+/−/+ embryos, whereas the transcriptional activity of Hes7 was slightly up-regulated (Fig. 4). This suggests that Hes7 3' UTR maintains the proper stability of Hes7 mRNA. It is not likely that the rate at which Hes7 is degraded is increased in Hes7−/− embryo because the primary structure of Hes7 protein is not altered by mutation in the Hes7 gene. Thus, the efficacy of export from the nucleus to the cytoplasm or translation of Hes7 mRNA may be reduced by abnormal 3' UTR. Further studies are needed to clarify the role of 3' UTR.

### Methods

#### Generation of Hes7+/−/+ knock-in mice.

We used mutated loxp sequences of lox71, lox2272 and lox2272-2272, respectively, in the presence of Cre recombinase17. The targeting vector was constructed by inserting lox71, PGK-neo, PGK-tk, and lox2272 just after the stop codon of Hes7, and it was transfected to embryonic stem (ES) cells (T2C)17. Hes7+/−/- clones were selected by using 250 µg/ml G418 (Nacalai tesque, Japan), and their genotypes were checked with Southern blotting. Whole placed lox66 and 2272 at the 's end and lox2272 at the 3' end of the fragments of the human dystrophin gene in the intron. We took 11 bp of the 3' end of exon 74, the consequent intron 74 (20 kb), and 10 bp of the 5' end of the exon 75 of the human dystrophin gene17. Vectors for the 10- and 5-kb introns were generated by deleting the middle part of the human dystrophin intron. The intron vectors were transfected into the Hes7+/−/- clone with the cre expression vector, and the Hes7+/−/- and Hes7−/−/- clones were selected by using 0.2 µM of FIAU (Wako, Japan). The genotypes were checked with Southern blotting and PCR. We confirmed Hes7 coding, introns, and the junction of the Hes7 gene and the exogenous fragment by sequencing. We inserted the Hes7+/−/-, Hes7+/−/-, and Hes7−/−/- ES cells into the blastocysts of CD1 mice, and generated chimeric mice, which were crossed with CD1 mice to generate Hes7+/−/-, Hes7−/−/- and Hes7+/−/+ mice. Hes7+/−/+ mice were intercrossed. Genotyping was checked with the following primers of Hes7_F GAGCAATGTGGTGACGTAGTTGT, Hes7_R TCTTACCTTCCTCCTGTC and Lfng_Rv CCGGAGGTGAGCACTGAG, and Gapdh_Rv TTCAATGATGACCGTGTC.

### 3’-rapid amplification of cDNA ends (3’ RACE).

Total RNAs were extracted from individual Hes7+/−/+ embryos at E10.5. cDNAs were synthesized from total RNAs by using SuperScriptII Reverse Transcriptase (Invitrogen) with oligo DT-3’-sites Adaptor Primer (Invitrogen) according to the manufacturer’s instructions. The 3’ fragments were amplified with KG6 primers under conditions of 95°C for 5 min, and 32 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 10 min. The sequences of the primers were: Primer_R1 GTGCCATGATGGGCTGTC, Primer_R2 ATGCCATGATGGCTGTC, and Primer_R3 GCGGCATGTCGTTGATGTGGATAAGCAG, Gapdh_Rv TTCAATGATGACCGTGTC.

#### Skeletal preparation.

The cartilages of newborn mice were stained with alcin blue and their bones were stained with alizarin red, after being fixed in 95% ethanol.

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Author contributions
T.F., T.M. and Y.N. performed most of the experiments. T.M. and Y.S. performed the mathematical analyses in supplementary note and the statistical analyses. M.S. and K.K. generated knock-in mice. T.M., Y.N. and Y.B. conceived and designed the experiments. Y.B. wrote the manuscript. All authors reviewed the manuscript.

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