Production of Wnt4b by floor plate cells is essential for the segmental patterning of the vertebral column in medaka

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SUMMARY
The floor plate is a key organizer that controls the specification of neurons in the central nervous system. Here, we show a new role of the floor plate: segmental pattern formation of the vertebral column. Analysis of a spontaneous medaka mutant, fused centrum (fsc), which exhibits fused centra and the absence of the intervertebral ligaments, revealed that fsc encodes wnt4b, which was expressed exclusively in the floor plate. Moreover, mosaic analyses showed the requirement of floor plate cells for the segmentation of the vertebral column. Taken together, our data allow the conclusion that production of Wnt4b by floor plate cells is essential for the segmental patterning of the vertebral column.

INTRODUCTION
In the process of the embryogenesis, specialized cells (or organs) act as organizing centers for adjacent tissues. The floor plate, a row of distinctive cells located at the ventral midline of the neural tube, is an important organizing center in the central nervous system. It is well known that the floor plate controls neural differentiation and axonal trajectories in the neural tube by secreting of Sonic hedgehog (Shh) and Netrin (Strähle et al., 2004; Placzek and Briscoe, 2005). Shh is a morphogen that induces motoneurons and different classes of interneurons along the dorsoventral axis according to its gradient, which is highest at the most ventral portion of the neural tube. Netrin 1 is the axon guidance molecule that acts as an attractant for the growing axons of the interneuron. These functions of the floor plate as the organizing center appear to be conserved from fish to mammals (Appel, 2000; Strähle et al., 2004). However, floor plate cells also secrete other signaling molecules, such as Wnt (Parr et al., 1993; Liu et al., 2000), indicating that these cells play unknown roles in the development of adjacent tissues.

In the present study, we analyzed a spontaneous medaka mutant, fused centrum (fsc), which exhibits fused centra and the absence of the intervertebral ligaments. In fsc mutants, we found abnormal conversion of the intervertebral ligament cells into osteoblasts. Positional cloning revealed that fsc encoded wnt4b, which was expressed exclusively in the floor plate during normal embryogenesis. In fsc mutants, we found that a DNA fragment was inserted in the promoter region of wnt4b gene, and wnt4b expression was not detected in the floor plate. We established the transgenic medaka lines, in which the exogenous genomic DNA fragment encoding wnt4b gene was stably integrated into the fsc genome, and succeeded to rescue the fish from the fsc phenotype.
amplified a 3.2 kb genomic fragment by using the subcloned genomic fragment as a template and appropriate primers (wnt4b-L2-HindIII, 5’-GCAAGCTTCCTCACTCGTTCCTCCC-3’; wnt4b-R1-SalI, 5’-GGCTCGAGACTGTTGCTGTCCTC-3’) and cloned it into HindIII-SalI sites of the pEGFP-1 vector (CLONTECH). This vector was designated as pEGFP1-wnt4b.

The establishment of transgenic lines was performed as previously described (Inohaya et al., 2007). The circular pEGFP1-wnt4b in phosphate-buffered saline was injected at a concentration of 17 ng/μl into the cytoplasm of 1-cell-stage embryos. For the establishment of transgenic lines, we used the fertilized eggs from the medaka Qurt line.

**osterix-EGFP transgenic medaka**

According to a previous report (Renn and Winkler, 2009), we amplified a genomic fragment encompassing the osterix promoter region from the medaka Hd-rf fosmid DNA (NRBP Medaka; http://www.shigen.nig.ac.jp/medaka/) by using the appropriate primers (osterix-Xhol-L, 5’-ACTCTGAGTCAAGTTGCAATGTC-3’; osterix-SalI-R, 5’-AGTCTGACCCCTAGGAAAATCTTCAAG-3’). The amplified fragment was cloned into the pGEM-T Easy vector (Promega). We then cloned a 4.2 kb DNA fragment into SacII-Xhol sites of the pEGFP-1 vector (CLONTECH). This vector was designated as pEGFP1-osterix. The circular pEGFP1-osterix in medaka Ringer’s solution was injected at a concentration of 2.5 ng/μl into the cytoplasm of 1-cell-stage embryos. For the establishment of transgenic lines, we used the fertilized eggs from the medaka Cab line.

**Transgenic rescue**

We used an 8.4 kb genomic fragment encompassing the wnt4b gene as a transgene. This transgene had a 3.2 kb wnt4b promoter region and a 2.9 kb 3’ non-coding region of the wnt4b gene, including a 3’ end of the cdc42 gene, a neighbor gene of wnt4b. We amplified a genomic fragment encompassing the wnt4b gene from the genomic DNA of the medaka Qurt line by using the appropriate primers (wnt4b-L2, 5’-TGCTCACTCGTTCCTCC-3’; wnt4b-cdc42-R, 5’-GATGGAGCCATACTGGAGACG-3’). The amplified fragment was cloned into the pGEM-T Easy vector (Promega). We then subcloned a reporter gene, which expresses EGFP under the control of a zebrafish α-crystallin promoter (Kurita et al., 2003), into the SacI site on the 3’ side of the 8.4 kb transgene [pW4b(8.4k)+cryEGFP].

The circular pW4b(8.4k)+cryEGFP in phosphate-buffered saline was injected at a concentration of 5 ng/μl into the cytoplasm of 1-cell-stage embryos. For the establishment of transgenic rescue lines, we used the fertilized eggs from the homozygous fsc mutant. After the injection, the embryos were cultured in medaka Ringer’s solution at 30°C until the desired developmental stages and were then checked for EGFP fluorescence under a fluorescence stereomicroscope (Leica, MZ FLIII). We selected the specimens in which transient EGFP expression was observed fluorescence under a fluorescence stereomicroscope (Leica, MZ FLIII). We selected the specimens in which transient EGFP expression was observed fluorescence under a fluorescence stereomicroscope (Leica, MZ FLIII). We selected the specimens in which transient EGFP expression was observed fluorescence under a fluorescence stereomicroscope (Leica, MZ FLIII). We selected the specimens in which transient EGFP expression was observed fluorescence under a fluorescence stereomicroscope (Leica, MZ FLIII). We selected the specimens in which transient EGFP expression was observed fluorescence under a fluorescence stereomicroscope (Leica, MZ FLIII). We selected the specimens in which transient EGFP expression was observed fluorescence under a fluorescence stereomicroscope (Leica, MZ FLIII). We selected the specimens in which transient EGFP expression was observed fluorescence under a fluorescence stereomicroscope (Leica, MZ FLIII). We selected the specimens in which transient EGFP expression was observed fluorescence under a fluorescence stereomicroscope (Leica, MZ FLIII). We selected the specimens in which transient EGFP expression was observed fluorescence under a fluorescence stereomicroscope (Leica, MZ FLIII). We selected the specimens in which transient EGFP expression was observed fluorescence under a fluorescence stereomicroscope (Leica, MZ FLIII).

**RESULTS AND DISCUSSION**

The **fused centrum** shows a defect in the segmental patterning of vertebral column

A spontaneous recessive mutant, fused centrum (fsc), or the so-called ‘Dharma-medaka’ in Japanese, has been bred among Japanese medaka enthusiasts for a long time. Homozygous mutants of fsc are viable and fertile and are easily distinguishable from the wild-type fish at a glance because the fsc mutant has a characteristically shortened body length (Fig. 1A,B). In the wild-type medaka, the vertebral column shows a metameric structure of the vertebrae. Each vertebra is composed of 3 elements: a centrum, a neural arch and a hemal arch; as well, an intervertebral ligament is seen between adjoining centra (Fig. 1C) (Inohaya et al., 2007). In adult fsc specimens, the vertebrae fused together and hence, these intervertebral ligaments were lost (Fig. 1D). It has been suggested that the reduced body length of fsc mutants is due to the loss of the intervertebral ligaments because the teleost intervertebral region acts as a growth center for the centrum. Our previous report shows that there are two types of osteoblastic cell, the osteoblast and the intervertebral ligament cell, in the intervertebral region and that the active matrix formation and mineralization proceeds remarkably at the anterior and posterior ends of each centrum by the osteoblast (Inohaya et al., 2007).

To clarify how the abnormal vertebral column is formed, we examined the mineralization pattern of vertebrae during fsc development. In the wild-type and fsc embryos, the mineralization of the vertebral column started at day 5 after fertilization (stage 37), when naturally fertilized eggs were cultured at 30°C. In the day-6 fsc embryos (stage 39), the centra fused at the ventral part of each developing centrum (Fig. 1E,F), suggesting that the segmental pattern of the vertebral column was already disrupted at the early stage of vertebral mineralization. In the fsc larvae, most of vertebrae had completely fused and the intervertebral ligaments were lost (Fig. 1G,H). These results indicate the possibility that the intervertebral ligaments are congenitally lacking in the fsc mutant. To confirm this possibility, we performed the histological analysis of the fsc larva. In fact, the histological sections revealed that there was not a vestige of the intervertebral ligament at the fused centra of the fsc larva (Fig. 1I,J). This result strongly suggests that, in the fsc mutant, the centra fuse owing to the absence of the intervertebral ligaments and not to the secondary mineralization of the intervertebral regions.

Although the fsc mutant showed an abnormality in the segmental pattern of its vertebral column, the segmental patterns of intersegmental vessels (Fig. 2A,B) and the axon trajectories of motoneurons (Fig. 2C,D) were normal in the fsc mutant. These results suggest that fsc functions specifically in the formation of the vertebral column.
The intervertebral ligament cells are converted into the osteoblasts in the fsc mutant

To define the fsc phenotype, we examined the development of osteoblast progenitors in the fsc mutant. The twist-EGFP transgenic medaka, which expresses the enhanced green fluorescent protein (EGFP) under the control of the twist promoter, is a powerful tool for tracing the behavior of progenitors for osteoblasts and intervertebral ligament cells (Inohaya et al., 2007). We then generated an fsc line carrying the twist-EGFP transgene (fsc twist-EGFP) and examined the alkaline phosphatase (ALP) activity, which is a typical marker of medaka osteoblasts, in the EGFP-positive osteoblast progenitors. In the wild-type twist-EGFP larvae, the EGFP-positive cells were observed on the outer surface of the notochord and in the centrum region (anterior part of each myotome), where they differentiated into the ALP-positive osteoblasts, whereas no ALP activity was detected in EGFP-positive cells located in the intervertebral region, i.e. the progenitors of intervertebral ligament cells (Fig. 3A,B) (Inohaya et al., 2007). By contrast, in the fsc twist-EGFP mutants, the EGFP-positive cells were observed on the outer surface of the notochord (Fig. 3C); however, the ALP-positive osteoblasts were detectable in the defective intervertebral regions as well as in the centrum regions (Fig. 3D). Moreover, we established an fsc line carrying the osterix-EGFP transgene (fsc osterix-EGFP) and analyzed the osteoblast development in this line. osterix is reported as a useful marker for early and mature osteoblasts in medaka and zebrafish (Renn and Winkler, 2009; Spoorendonk et al., 2008). In the wild-type osterix-EGFP larvae, the osterix-EGFP-positive osteoblasts were observed at the centra and vertebral arches, but not at the intervertebral regions (Fig. 3E); however, in the fsc osterix-EGFP mutants, the EGFP-positive osteoblasts were aberrantly detected at the defective intervertebral regions, i.e. the fused centra (Fig. 3F). These results suggest that the progenitors of intervertebral ligament cells are differentiated into the osteoblasts in the fsc mutant.

Positional cloning reveals that the fsc encodes wnt4b

To clarify the molecular mechanism of segmental patterning of the vertebral column, we performed positional cloning of the candidate gene responsible for the fsc mutant. The fsc locus was genetically mapped to linkage group (LG) 16 and was positioned within a genetic interval of about 89 kb. By performing a genomic PCR reaction, we found an insertion fragment in the 5' non-coding region of the wnt4b gene (Fig. 4A). Sequence analysis revealed that a 5.5 kb fragment had been inserted in the vicinity of the presumed TATA box sequence of the wnt4b gene (Fig. 4B). In addition, the sequence of this insertion was similar to that of a Pol-like protein, which contains a reverse transcriptase domain (data not shown).

The putative amino acid sequence of medaka wnt4b showed highest similarity to the Wnt4 subgroup. In addition, the sequence of medaka wnt4b was clearly different from that of medaka wnt4 (medaka wnt4 will hereafter be referred to as medaka wnt4a)
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(Yokoi et al., 2003) and was most related to that of zebrafish wnt4b (see Fig. S1 in the supplementary material). Medaka wnt4b was expressed exclusively in the floor plate during embryogenesis, just as in the case of zebrafish wnt4b (Liu et al., 2000). In the wild-type medaka, wnt4b expression in the floor plate was first detected around stage 25 (data not shown) and was then observed distinctly in the day-3 embryos (stage 29; Fig. 4C-E). Also, faint expression of wnt4b was observable in the otic vesicles of day-3 embryos (Fig. 4C). By contrast, wnt4b expression in the floor plate was not detectable in the fsc mutant (Fig. 4F).

The floor plate markers such as netrin 1b (ntn1b) and sonic hedgehog (shh) were expressed normally in the fsc mutant (see Fig. S2A,B in the supplementary material). In addition, wnt4a was also expressed normally in the fsc embryos. In the day-3 embryos, the expression pattern of wnt4a overlapped that of wnt4b in the floor plate and in the otic vesicles (see Fig. S2C in the supplementary material). Although this data suggested that wnt4a might be redundant with wnt4b in these organs, the fsc phenotype was not exacerbated by the knockdown of wnt4a by using morpholino oligomers (data not shown). Moreover, we established a stable transgenic medaka that expressed fluorescent EGFP under the control of the wnt4b promoter region (wnt4b-EGFP). We found that a 3.2 kb wnt4b promoter region contained the cis-regulatory elements necessary for expression in the floor plate but not in the otic vesicles (see Fig. S3A-C in the supplementary material). The EGFP expression was also detected in the floor plate cells of the fsc wnt4b-EGFP specimen (see Fig. S3D in the supplementary material), indicating that the transcriptional pathway of wnt4b gene was normally functioned in the fsc mutant. Morphological distinctions of the EGFP-positive floor plate cells were not observed between the wild-type and fsc wnt4b-EGFP larvae (see Fig. S3C,D in the supplementary material). These results suggest

Fig. 3. The osteoblast development in fsc mutants. (A–D) Development of osteoblast progenitors in the twist-EGFP transgenic medaka. Horizontal sections of the wild-type twist-EGFP embryo (A,B) and fsc twist-EGFP mutant (C,D). Anterior to the left. (A,C) Expression of twist-EGFP (green). (B,D) Sections adjacent to those in A and C, respectively, and stained for ALP activity (red). The dashed white lines indicate myotome boundaries. (E,F) Development of osteoblasts in the osterix-EGFP transgenic medaka larvae. Lateral views of wild-type osterix-EGFP (E) and fsc osterix-EGFP mutant (F) larvae. Anterior to the left. The square bracket indicates the defective intervertebral region in the fsc mutant. ct, centrum; IVR, intervertebral region; dIVR, defective IVR; myo, myotome; n, notochord; na, neural arch. Scale bars: 20 μm in A–D; 50 μm in E,F.

Fig. 4. fsc encodes wnt4b. (A) An insertion fragment is found in the 5′ non-coding region of the fsc wnt4b gene by using genomic PCR with the primer sets figured in B. (B) A 5.5 kb insertion fragment (gray box) is located in the vicinity of the presumed TATA box sequence (red box) of the fsc wnt4b gene. Yellow boxes indicate the duplicated genomic sequences. Green boxes and the blue box show two exons of the 5′ non-coding region and an exon of coding region, respectively. Arrows indicate the designed primers for the genomic PCR in A. (C) Expression of wnt4b in the wild-type embryo. Ventral view of a day-3 embryo. Anterior to the top. (D) Higher magnification of a part of C. Lateral view; anterior to the left. (E) Transverse section of the day-3 embryo expressing wnt4b in the floor plate. (F) Expression of wnt4b in the fsc embryo. Ventral view of a day-3 embryo. Anterior to the top. fp, floor plate; ms, muscle; n, notochord; nt, neural tube; ov, otic vesicle. Scale bars: 250 μm in C,F; 100 μm in D; 50 μm in E.
that the floor plate was formed in the fsc mutant and that the 5.5 kb insertion specifically blocked the wnt4b expression in the floor plate during fsc embryogenesis.

**Expression of wnt4b in the floor plate is responsible for the fsc phenotype**

To verify that disappearance of wnt4b transcripts from the floor plate was responsible for the fsc phenotype, we established transgenic medaka to rescue the fish from the fsc phenotype by using an 8.4 kb genomic fragment encompassing the wnt4b gene as a transgene (Fig. 5A). This transgene had a 3.2 kb wnt4b promoter region that contained the cis-regulatory elements necessary for expression in the floor plate but not in the otic vesicles (see Fig. S3 in the supplementary material). In addition, we inserted a reporter gene that expressed EGFP under the control of a zebrafish αA-crystallin promoter into the 3' side of the 8.4 kb transgene (Fig. 5A). We designed this reporter gene system to distinguish the transgenic specimens from other siblings easily by observing EGFP expression in their lens under a fluorescence stereomicroscope and this system was very helpful for screening for transgenic specimens in the present study.

The established transgenic rescue lines were viable and fertile and exhibited Mendelian inheritance of the transgene, indicating that the exogenous DNA had integrated itself at a single locus. According to the results obtained from genomic PCR analysis, the transgenic specimens had the exogenous wnt4b gene on a homozygous fsc genomic background (Fig. 5B). In addition, the wnt4b expression in the floor plate was detectable in the transgenic specimens that had the EGFP-positive lens (Fig. 5C,D). Then we investigated the skeletal structures of the sibling embryos (n=433) obtained from the mating between the heterozygous transgenic rescue specimens (TgRe+/–; fsc+/–) and non-transgenic fsc mutants (fsc–/–). The transgenic larvae showed a normal segmental pattern in their vertebral column [100% (n=221); Fig. 5F], whereas their siblings showed the fused centra and the defective intervertebral ligaments [100% (n=212); Fig. 5E]. These findings indicate that exogenous wnt4b functioned in the floor plate of the transgenic embryos and rescued these transgenic specimens from the fsc phenotype.

**The floor plate cells are required for the segmental patterning of the vertebral column**

Finally, we performed a mosaic analysis experiment to demonstrate that the floor plate participates in the vertebral column formation (see Fig. S4A in the supplementary material). We first confirmed that rhodamine-labeled wild-type cells contributed to the putative floor plate cells in homozygous fsc mutants (Fig. 6A). In addition, the fsc phenotype of the vertebral column was reversed to normal beneath the putative floor plate cells, which were derived from the transplanted wild-type cells (n=5; Fig. 6B,C). We then performed the mosaic analysis experiment using the wnt4b-EGFP transgenic line (see Fig. S3 in the supplementary material) as the wild-type donor to visualize that the transplanted wild-type cells differentiated into the floor plate cells expressing the wnt4b gene (see Fig. S4B in the supplementary material). As a result, we found that the fsc phenotype was reversed beneath the donor-derived floor plate cells expressing EGFP (n=5; Fig. 6D-F). By contrast, the EGFP-negative transplanted cells (non-floor plate cells) were not
Fig. 6. Mosaic experiment reveals that the floor plate cells are essential for the vertebral column segmentation. Lateral views of day-6 embryo. Anterior to the left. (A) The rhodamine-labeled donor wild-type cells in the recipient fsc embryo are detected in red (arrowheads). (B) The vertebral column of the specimen was stained with Calcein (green). (C) A merged image of A and B. The fsc phenotype is reversed to normal beneath the transplanted wild-type cells (red; arrowheads). (D) The rhodamine-labeled wild-type cells, which are derived from a wnt4b-EGFP transgenic embryo, are detected in red (arrowheads). (E) The vertebral column of the specimen was stained with Calcein (green). The floor plate cells expressing wnt4b-EGFP are also observed in green (arrows). (F) A merged image of D and E. The fsc phenotype is reversed to normal beneath the double-positive cells (yellow; arrows). The EGFP-negative transplanted cells are not able to rescue the embryo from the fsc phenotype (arrowhead). The square bracket indicates rescued segmental vertebrae in the fsc mutants. vc, vertebral column. Scale bar: 100 μm.

References


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