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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. In *fsc* mutants, we found that *wnt4b* expression was completely lost in the floor plate and that abnormal conversion of the intervertebral ligament cells into osteoblasts appeared to cause a defect of the intervertebral ligaments. The establishment of the transgenic rescue lines and mosaic analyses allowed the conclusion to be drawn that production of *wnt4b* by floor plate cells is essential for the segmental patterning of the vertebral column. Our findings provide a novel perspective on the mechanism of vertebrate development.

KEY WORDS: Wnt, Floor plate, Medaka, Vertebral column, Segmental patterning, Teleost

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. 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.

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MATERIALS AND METHODS

Fish

Homozygous *fsc* mutants were obtained from a dealer. The siblings obtained from the mating of homozygous *fsc* mutant and wild-type Qurt lines were used as heterozygous *fsc* specimens. The inbred strain HNI was used for genetic mapping. The naturally fertilized eggs were cultured in medaka Ringer's solution at 30°C. The embryos were staged according to Iwamatsu (Iwamatsu, 1994) together with some morphological characteristics employed for our criteria.

Mapping

The homozygous *fsc* mutant was mated with the HNI, and then the F1 hybrids were intercrossed to generate a reference panel for mapping. The *fsc* locus was genetically mapped to LG16 by using the M markers (Kimura et al., 2004). Further examination was performed using a 478 F2 progeny panel of 956 meioses, and the *fsc* mutant was positioned within a genetic interval of about 89 kb. The mapping primers were designed by using the Medaka Genome Database and University of Tokyo Genome Browser Medaka database (UTGB/medaka database) (Ahsan et al., 2008). The candidate genes were predicted by using GENESCAN (http://genes.mit.edu/GENSCAN.html).

Skeletal staining

The skeletal staining with Alizarin Red, Calcein and Alizarin Complexon (ALC) was performed as previously described (Inohaya et al., 2007).

Histochemical method

The preparation of histological sections and alkaline phosphatase (ALP) staining were performed as previously described (Inohaya et al., 2007).

wnt4b-EGFP transgenic medaka

We amplified a genomic fragment encompassing the *wnt4b* promoter region from the genomic DNA of the medaka Qurt line by using the appropriate primers (*wnt4b*-L2, 5′-TGCCTCACCTGTTCCTTCCC-3′; *wnt4b*-R1, 5′-TGACAGGGGAGACAGTTGGC-3′). The amplified fragment was cloned into the pGEM-T Easy vector (Promega). We then

amplified a 3.2 kb genomic fragment by using the subcloned genomic fragment as a template and appropriate primers (*wnt4b*-L2-*Hin*dIII, 5′-GCAAGCTTGCCTCACCTGTTCCTTCCC-3′; *wnt4b*-R1-*Sal*I, 5′-GCGTCGACAGACTGTCGGTCTGTCTCTC-3′) and cloned it into *Hin*dIII-*Sal*I 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-rR fosmid DNA (NBRP Medaka; http://www.shigen.nig.ac.jp/medaka/) by using the appropriate primers (*osterix*-*Xho*I-L, 5′-ACTC-GAGTCAGTGCCATCAGAATGTCA-3′; *osterix*-*Sal*I-R, 5′-AGTCGA-CCCTGAGAAACAAAATCCTAAG-3′). The amplified fragment was cloned into the pGEM-T Easy vector (Promega). We then cloned a 4.2 kb DNA fragment into *Sac*II-*Xho*I 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′-TGCCTCACCTGTTC-CTTCCC-3′; *wnt4-cdc42*-R, 5′-GATGAGGCCATACTGGCAGC-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*A-crystallin* promoter (Kurita et al., 2003), into the *Sac*I 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 well and kept them as founders. We identified two germline-transmitting founders. The identified founders were then mated with non-transgenic individuals of the homozygous *fsc* mutant. F1 offsprings of each founder were maintained and analyzed in subsequent generations. We confirmed that these two transgenic lines were equally successful in rescuing the *fsc* phenotype.

To check the genotypes of the wild type and *fsc* mutant, we performed genomic PCR analysis by using appropriate primers (*wnt4b*-L1, 5′-TGAAGGAGGACCATTCTGGAG-3′; *wnt4b*-R1, 5′-TGACAGGGGA-GACAGTTGGC-3′; *wnt4b-fsc*-R2, 5′-CCCGAAGTCTGCCGTCAAAG-3′). To confirm the genotype of the transgenic rescue specimens, we performed genomic PCR by using the *wnt4b*-R1 primer and the F primer (5′-CACGACGTTGTAAAACGACGG-3′), which is specific to the pGEM-T Easy vector.

Mosaic experiment

Labeling of donor embryos and transplantation of embryonic shield were performed as previously described (Inohaya et al., 1999). The donor embryos of the medaka Qurt line or *wnt4b*-EGFP transgenic line were injected with 1.7% rhodamine-dextran (dextran, tetramethylrhodamine, molecular weight 10,000, neutral; Molecular Probes) at the 1-cell-stage, and they were then cultured in medaka Ringer's solution at 30°C until the early gastrula stage. The donor and host embryos were dechorionated with partially purified hatching enzyme (step 1) (Yasumasu et al., 1989) before the early gastrula stage. The embryonic shield cells of the labeled donor embryos were transplanted into the embryonic shield of the homozygous *fsc* mutant at the early gastrula stage. The donor and host embryos were cultured at 30°C until the hatching stage. Then, skeletal structures of the specimens were stained with Calcein (Inohaya et al., 2007). The donor cells contributing to the floor plate and the mineralization of vertebral column were detected by observation with 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* larvae (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.

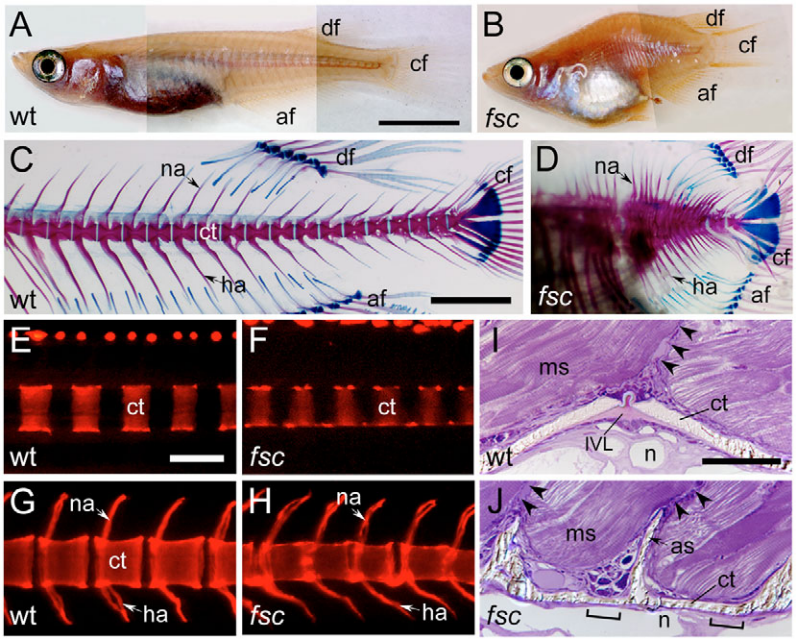

Fig. 1. Phenotype of the *fsc* mutant. (**A**,**B**) Lateral views of wild-type (A) and *fsc* mutant (B) adult male medaka. Anterior to the left. (**C**,**D**) Skeletal structures of wild-type (C) and *fsc* mutant (D) adult male medaka. Bony tissues were stained with Alizarin Red. Lateral view, anterior to the left. (**E-H**) In vivo observation of the vertebral column in the wild-type (E,G) and *fsc* (F,H) specimens. The vertebral column was stained with Alizarin Complexon (ALC). Lateral view, anterior to the left. (E,F) Day-6 embryos. (G,H) Day-5 larvae. (**I**,**J**) Horizontal sections of the wild-type (I) and *fsc* mutant (J) larvae. Anterior to the left. The sections were stained with Toluidine Blue. The arrowheads indicate myotome boundaries. The deformation of the proximal region of the neural and hemal arches (as) was observed in the *fsc* mutants. af, anal fin; cf, caudal fin; ct, centrum; df, dorsal fin; ha, hemal arch; IVL, intervertebral ligament; ms, muscle; n, notochord; na, neural arch. Scale bars: 5 mm in A,B; 2 mm in C,D; 100 μm in E-H; 50 μm in I,J.

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.

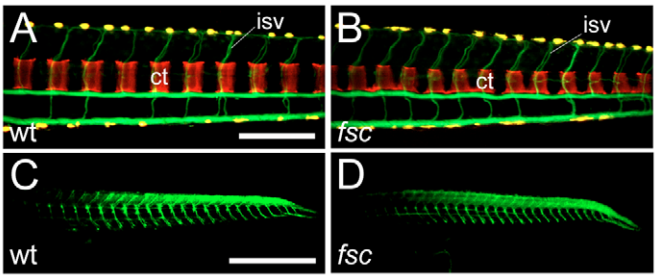

Fig. 2. The segmental patterns of intersegmental vessels and axon trajectories of motoneurons in the *fsc* mutants. (**A**,**B**) Lateral views of the just-hatched larvae of the wild type (A) and *fsc* mutant (B). Anterior to the left. The blood vessels (green) are visualized in the α-*globin*-EGFP transgenic medaka. The vertebral column of the specimens are stained with ALC (red). (**C**,**D**) Lateral views of the day-4 embryos of the wild type (C) and *fsc* mutant (D). Anterior to the left. Axon trajectories of CaP motoneurons are detected by using anti-acetylated tubulin antibody (green). ct, centrum; isv, intersegmental vessels. Scale bars: 250 μm in A,B; 500 μm in C,D.

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*)

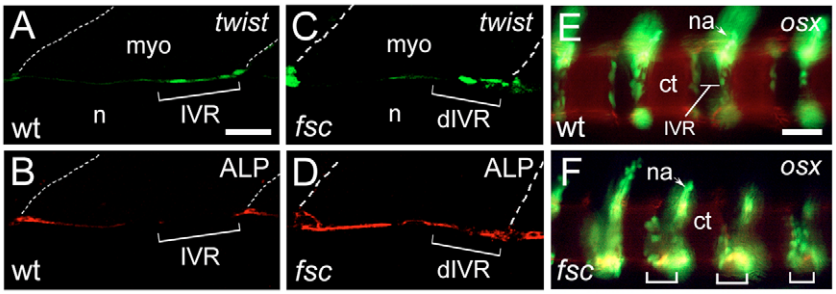

Fig. 3. The osteoblast development in *fsc* mutants. (**A-D**) Development of osteoblast progenitors in the *twist*-EGFP transgenic medaka. Horizontal sections of the wild-type *twist*-EGFP embryo (A,B) and *fsc twist*-EGFP mutant (C,D). Anterior to the left. (A,C) Expression of *twist*-EGFP (green). (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.

(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 *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

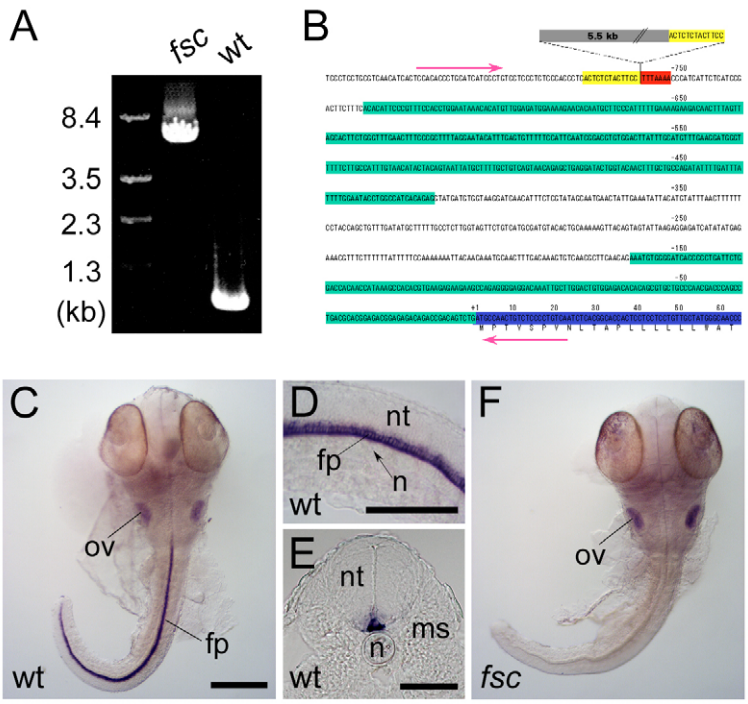

Fig. 4. *fsc* encodes *wnt4b*. (**A**) An insertion fragment is found in the 5′ non-coding region of the *fsc wnt4b* gene by using genomic PCR with the primer sets figured in B. (**B**) A 5.5 kb insertion fragment (gray box) is located in the vicinity of the presumed TATA box sequence (red box) of the *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.

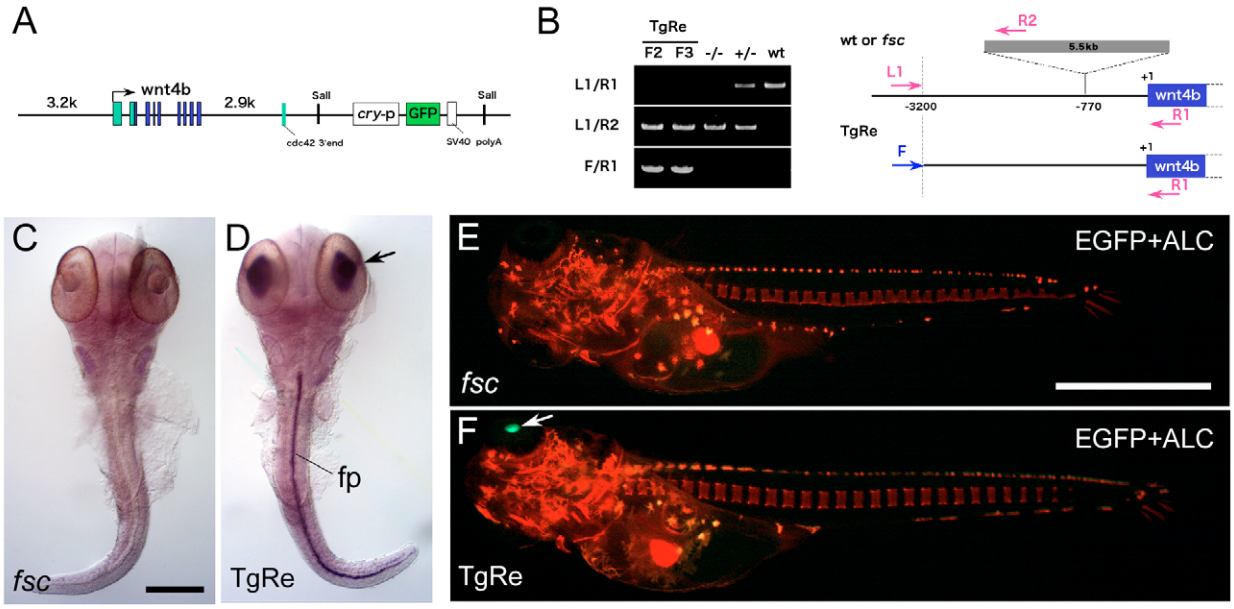

Fig. 5. Transgenic medaka reveals that the *wnt4b* expression in the floor plate rescues larvae from the *fsc* phenotype. (**A**) Scheme of the transgene construct. (**B**) The genotype of the transgenic rescue medaka was confirmed by PCR analysis. The primers were designed for the promoter region of the *wnt4b* gene, as represented in the scheme on the right. The PCR results reveal that the transgenic rescue specimens have the *fsc* genetic background. TgRe, transgenic rescue medaka; F2, F2 offspring; F3, F3 offspring; –/–, homozygous *fsc*; +/–, heterozygous *fsc*; wt, wild type. (**C**,**D**) Expression of EGFP and *wnt4b* in day-3 embryos. Ventral views of an *fsc* embryo (C) and transgenic rescue embryo (D). The *wnt4b* expression is detectable in the floor plate (fp) of the transgenic rescue embryos (F3 offspring), but not that of their siblings. (**E**,**F**) Merged images of EGFP expression and ALC staining. Lateral views of the hatched larvae (F3 offspring), anterior to the left. Although the sibling of the transgenic rescue specimen shows the fusion of centra (E), the transgenic rescue specimen shows normal segmental pattern of the vertebral column (F). Arrows in D and F indicate the EGFP-positive lens in the transgenic rescue specimen. Scale bars: 250 μm in C,D; 1 mm in E,F.

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 α*A-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

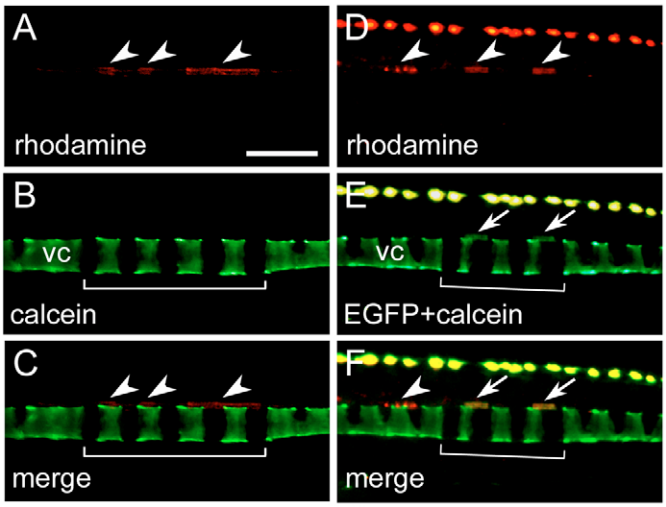

Fig. 6. Mosaic experiment reveals that the floor plate cells are essential for the vertebral column segmentation. Lateral views of day-6 embryo. Anterior to the left. (**A**) The rhodamine-labeled donor wild-type cells in the recipient *fsc* embryo are detected in red (arrowheads). (**B**) The vertebral 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.

able to rescue the fish from the *fsc* phenotype (n=4 out of 5; Fig. 6F). Based on all data, we conclude that *wnt4b* is the gene responsible for the *fsc* mutant and that the Wnt4b-producing floor plate is essential for the segmental patterning of the vertebral column.

Previous studies on *Wnt4*-mutant mice show that Wnt4 signaling is essential for nephrogenesis and female sexual development (Stark et al., 1994; Vainio et al., 1999). Additionally, Wnt4 is expressed in the floor plate during mouse embryogenesis, and a previous report indicates the possibility that mouse Wnt4 acts as a guidance molecule for commissural axons (Parr et al., 1993; Lyuksyutova et al., 2003). However, it had not been previously reported whether or not mouse Wnt4 signaling participates in the vertebral column formation. Therefore, our data provide a new insight into the molecular mechanism of vertebral column formation. The mechanism of segmentation of the vertebral column is not clear in the teleost. In higher vertebrates, such as birds and mammals, the segmental patterning of vertebral column is exclusively dependent on the embryonic somite segmentation because the vertebral column is formed by a process termed resegmentation; the posterior part of one somite fuses to the anterior part of the consecutive somite to form vertebrae (Bagnall et al., 1988; Aoyama and Asamoto, 2000). Therefore, the defects in the somite segmentation strongly affect the segmental patterning of the vertebral column (Saga and Takeda, 2001). In the zebrafish *fss*-type mutants, however, the segmental patterning of vertebral column develops normally, whereas the somite segmentation is abnormal in the early embryonic stage (van Eden et al., 1996). According to the leaky resegmentation model in zebrafish (Morin-Kensicki et al., 2002), there is a possibility that the segmental patterning of vertebral column is controlled by some unknown segmental mechanisms in the teleost development. Although the Wnt4b signaling pathway is a convincing candidate of the unknown segmental mechanism, *wnt4b* is not expressed in a segmental pattern in the floor plate of medaka and zebrafish. The progenitors for both osteoblasts and intervertebral ligament cells are candidates for the target of Wnt4b signaling as our finding shows that the *wnt4b* gene might control the fate determination of these progenitors. Fused vertebrae have been reported among many fish species. The phenotype of *fsc* is similar to that of medaka *fused* (*fu*) mutants (Aida, 1930; Ogawa, 1965; Tomita, 1992). The so-called 'Ryukin' of goldfish (*Carassius auratus*) also has fused vertebrae, and the calcification pattern of their vertebral column during embryogenesis is similar to that of *fsc* (our unpublished results). Analysis of these mutants would provide a more complete perspective on the Wnt4b signaling pathway.

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Competing interests statement
The authors declare no competing financial interests.

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