

# Fgf22 regulated by Fgf3/Fgf8 signaling is required for zebrafish midbrain development

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## Summary

Fibroblast growth factor (Fgf) signaling plays important roles in various developmental processes including brain development. Here, we identified zebrafish *fgf22* predominantly expressed in the posterior midbrain and anterior midbrain–hindbrain boundary (MHB) primordia during early embryonic brain development. To examine roles of Fgf22 in midbrain development, we analyzed *fgf22* knockdown embryos. The *fgf22* morphants were defective in proper formation of the MHB constriction and the midbrain. The knockdown of *fgf22* caused decreased cell proliferation in the midbrain, expanded expression of roof plate and tegmental marker genes, and decreased expression of tectal marker genes, indicating that Fgf22 is required for cell proliferation, roof plate formation, and tectum specification in the midbrain. Fgf receptor 2b (Fgfr2b), a potential receptor for Fgf22, was also required, indicating that Fgf22 signaling is mediated through Fgfr2b. The floor plate and the MHB are crucial for the dorsoventral patterning of the midbrain through Hedgehog

(Hh) and Fgf signaling, respectively. The *fgf3/fgf8* double morphant phenotype was essentially similar to that of *fgf22* morphants, whereas the phenotype caused by inhibition of Hh signaling was not. *fgf3* and *fgf8* were expressed earlier than *fgf22* in the MHB primordium and Fgf3/Fgf8 signaling was required for *fgf22* expression in the posterior midbrain. Furthermore, *fgf22* partially rescued the *fgf3/fgf8* double morphant phenotype. The present results indicate Fgf22 to be involved in midbrain development downstream of Fgf3 and Fgf8 in the MHB but not of Hh in the floor plate.

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Key words: Fgf, Fgf22, Fgf3, Fgf8, Zebrafish, Development, MHB, Midbrain, Regionalization, Proliferation

## Introduction

During early embryonic brain development in vertebrates, the neural plate is regionalized along the anteroposterior (A/P) and dorsoventral (D/V) axis. Patterning along the A/P and D/V axis of the neural tube is finely regulated by signals that emanate from adjacent tissues and/or from the neuroepithelium itself. The best characterized local organizing centers involved in the refinement of A/P and D/V patterns are the roof plate and floor plate, the anterior neural ridge, the zona limitans intrathalamica, and the isthmic organizer, also referred to as the midbrain–hindbrain boundary (MHB) (reviewed by Altmann and Brivanlou, 2001; Briscoe and Ericson, 2001; Liu and Joyner, 2001; Rhinn and Brand, 2001; Simeone, 2002; Wilson et al., 2002). Among them, the roof plate and floor plate are specialized structures that mark the dorsal and ventral midline of the neural tube, respectively, and are involved in D/V patterning. D/V patterning mechanisms have been best studied in the developing spinal cord and depend on the relative amount of a ventralizing factor, Sonic hedgehog (Shh), provided by the floor plate and notochord and dorsalizing factors, Bone morphogenetic proteins (Bmps), produced by the roof plate. In mice lacking *Shh* gene function, the nervous system shows abnormalities in the development of ventral midline structures like the floor plate and notochord and the differentiation of ventral cell types (Chiang et al., 1996). Conversely, the misexpression of *Shh* transforms cell fate specification, from dorsal to ventral cells, and induces

differentiation into ventral neuronal cell types in the dorsal region (Agarwala et al., 2001). On the other hand, the Bmps coordinate dorsal patterning of the neural tube and the generation of different dorsal neuronal cell types in the spinal cord. The disruption of the Bmp antagonist Noggin induces D/V patterning defects in the neural tube and ventral neurons are missing in noggin mutants (Alexandre and Wassef, 2005). These general mechanisms of D/V patterning are common to the spinal cord and midbrain. However, the exact functions of genes involved in D/V patterning of the midbrain and the interactions between these genes are still not well understood. Furthermore, several observations suggest that midbrain D/V patterning requires additional signals.

Fibroblast growth factors (Fgfs) make up a large family comprising 22 members in mammals. Among them, Fgf22 is a member of the Fgf7 subfamily (Itoh and Ornitz, 2004). Fgf signaling is mediated by Fgf receptor (Fgfr) proteins, which belong to a family of tyrosine kinase-containing transmembrane proteins that bind to Fgf molecules. The *Fgfr* gene family comprises four members, *Fgfr1–Fgfr4* (Itoh and Ornitz, 2004). Fgf22 preferentially binds to a product of the *Fgfr2b* gene (Zhang et al., 2006). Here, we identified zebrafish *fgf22* predominantly expressed in the posterior midbrain and anterior MHB primordia during early embryonic brain development. Fgf22 was critical for cell proliferation, the formation of the roof plate, and the specification of the tectum through Fgfr2b in the midbrain. In

addition to the floor plate, the isthmic organizer is crucial for the patterning of the midbrain through the production of several secreted molecules (Alexandre and Wassef, 2005). However, less is known about the influence of the isthmic organizer on the midbrain D/V patterning. *fgf3* and *fgf8* were expressed at earlier stages than *fgf22* in the MHB primordium. Fgf3/Fgf8 signaling was required for *fgf22* expression in the posterior midbrain. The *fgf3/fgf8* double morphant phenotype was essentially similar to that of *fgf22* morphants, and partially rescued by *fgf22*. However, the phenotype caused by inhibition of Hedgehog (Hh) signaling in the floor plate differed from that of *fgf22* morphants. The present results indicate that Fgf22 regulated by Fgf3/Fgf8 signaling but not by Hh signaling is involved in the formation of the roof plate and the specification of the tectum through Fgfr2b in the midbrain. The present findings should provide new insights into roles of Fgf signaling in midbrain development.

## Materials and Methods

### Fish maintenance

Zebrafish (*Danio rerio*) were maintained, referring to *The Zebrafish Book* (Westerfield, 1995). Embryos were obtained by natural spawning and cultured at 28.5°C in Zebrafish Ringer's solution. The developmental stages of the embryos were determined by the hours post fertilization (hpf) and by morphological features, as described by Kimmel et al. (Kimmel et al., 1995).

### Isolation and characterization of zebrafish *Fgf22* cDNA

Zebrafish *fgf22* was identified by BLAST (Basic Local Alignment Search Tool, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) – searching zebrafish cDNA and genomic DNA sequences with the amino acid sequence of human FGF22. The full-length cDNA was isolated by polymerase chain reaction (PCR) with zebrafish embryonic cDNA as a template. The GenBank accession number for the *fgf22* cDNA is AB254028.

The positions of zebrafish *fgf22*, *bsg*, *hcn2*, and *polrmt* on chromosome 22 were obtained from the Ensembl Zebrafish Genome Browser ([http://www.ensembl.org/Danio\\_rerio](http://www.ensembl.org/Danio_rerio)). The map positions of human *FGF22*, *BSG*, *HCN2*, and *POLRMT* on chromosome 19 were obtained from LocusLink (<http://www.ncbi.nlm.nih.gov/genome/guide>).

Temporal expression profiles were determined by reverse transcription-polymerase chain reaction (RT-PCR) using the following primers (5' primer/3' primer): *fgf22*, 5'-CATCATGCCGACTGCTGTGCA-3'/5'-TGATGAAGTGTCCGGCTATGTG-3' (688 bp fragment) and zebrafish *elongation factor 1-a (ef1a)* (Miyake et al., 2005).

### Whole mount in situ hybridization and sectioning

Digoxigenin- or fluorescein-labeled RNA probes were synthesized by *in vitro* transcription using T7 or SP6 RNA polymerase. A 0.7-kb *fgf22* probe was synthesized using the full-length cDNA-containing plasmid. Other probes used were zebrafish *wnt1* (Kelly and Moon, 1995), *pax2.1* (Krauss et al., 1991), *otx2* (Mori et al., 1994), *eng2* (Ekker et al., 1992), *her5* (Müller et al., 1996), *Fgf8* (Reifers et al., 1998), *Fgf3* (Phillips et al., 2001), *nkx6.2* (Guner and Karlstrom, 2007), *pax7a* (Seo et al., 1998), *lmx1b.2* (Elsen et al., 2008), *bmp5* (Holzschuh et al., 2005), *meis2a* (Waskiewicz et al., 2001), and *msxb* (Ekker et al., 1997). Whole mount in situ hybridization was performed according to standard protocols and developed with BM Purple (Roche) and Fast Red (Roche).

Fixed embryos were transferred to 20% sucrose in PBS, mounted in OCT compound, and sectioned at 16 µm.

### Injection of morpholino oligonucleotides

Morpholino oligonucleotides (MOs) were synthesized by Gene-Tools, LLC (Corvallis, OR). MOs were diluted in Danieau buffer (Nasevicius and Ekker, 2000). The sequences of MOs used are as follows: *fgf22* exon 2/intron 2 splice-blocking MO1, 5'-ATGCGATGTACTACCGATCCGAAAAG-3'; *fgf22* exon 1/intron 1 splice-blocking *fgf22* MO2, 5'-AGCACTGTGTATCTACTCACTGTCA-3'; *fgfr2b* exon 7/intron 7 splice-blocking MO1, 5'-CCTGCTTTTTTACCTGGTATGACAA-3'; *fgfr2b* exon 7/intron 7 splice-blocking MO2, 5'-CCACGCTCCTGCTTTTTTACCTGGT-3'; and universal control MO, 5'-CCTCTTACCTCAGTTACAATTATA-3'. *fgf3* MO, *fgf8* MO, and *tp53* MO were reported previously (Maroon et al., 2002; Miyake et al., 2005; Gerety and Wilkinson, 2011; Miyake et al., 2012). *fgf22* MO1 (12 ng), *fgf22* MO2 (30 ng), *fgfr2b* MO1 (12 ng), *fgfr2b* MO2 (8 ng), or universal control MO (12 ng) was injected into zebrafish 2- to 4-cell embryos. *fgf3* MO (10 µg/µl) and *fgf8* MO (20 µg/µl) were injected at a volume of 0.15–0.25 nl into one- to two-cell embryos. *tp53* MO (13.4 µg/µl) was injected at 0.35–0.4 nl into 2-cell embryos.

To determine the efficacy of MOs, RNA was isolated from wild-type, *fgf22* MO1, *fgf22* MO2, *fgfr2b* MO1, or *fgfr2b* MO2-injected embryos. cDNA was amplified from the RNA by RT-PCR using the above primers and the following primers (5' primer/3' primer): *fgfr2b*, 5'-GAGCTCGGGCATAAACAGCT-3'/5'-CTGGAGGATAATCCGTCTCG-3' (176 bp fragment) and *fgfr2c*, 5'-GACGGCAGGTGTGAACACTA-3'/5'-CTGGAGGATAATCCGTCTCG-3' (182 bp fragment).

### RNA injection

The entire coding region of zebrafish *fgf22* cDNA was inserted into a vector, *pCS2+* (Turner and Weintraub, 1994). Capped *fgf22* mRNA was synthesized using a mMESSAGE mMACHINE kit (Ambion) from a linearized *pCS2+* containing *fgf22* cDNA. The mRNA was diluted to 10 ng/µl with water and injected in a volume of 1 nl into zebrafish 2- to 4-cell embryos.

### H3P antibody staining and TUNEL assay

Proliferating and apoptotic cells were detected using a rabbit polyclonal anti-phosphorylated histone H3 (H3P) (Upstate Biotechnology) antibody and the DeadEnd colorimetric detection kit (Promega), respectively (Miyake et al., 2005).

For cell counts, the stained embryos were embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim) and cut into 4-µm serial sections. The sections were counterstained with hematoxylin.

### Cyclopamine treatments

Cyclopamine (Toronto Chemical) (Incardona et al., 1998) was dissolved at 10 mM in 95% ethanol. Embryos, in their chorions, were incubated in cyclopamine diluted to 100 µM in Zebrafish Ringer's solution starting at the time points indicated. Control embryos were treated simultaneously with an equal volume of 0.95% ethanol (cyclopamine carrier) in Zebrafish Ringer's solution.

### Hydroxyurea–Aphidicholin (HUA) treatments

Mid-gastrula embryos (80% epiboly) were incubated in Zebrafish Ringer's solution containing 20 mM Hydroxyurea (Sigma–Aldrich), 150 µM Aphidicholin (Sigma–Aldrich), and 4% dimethyl sulfoxide (DMSO). Control embryos were treated simultaneously with an equal volume of 4% DMSO (HUA carrier) in Zebrafish Ringer's solution.

## Results

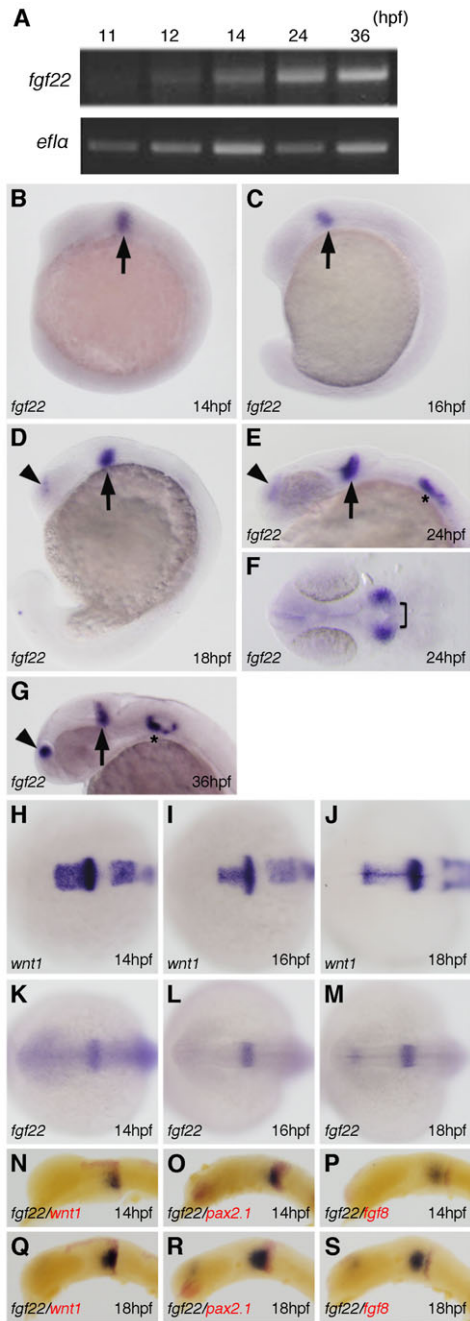
### Identification and characterization of zebrafish *fgf22*

Among vertebrates, amino acid sequences of most orthologous Fgfs are highly conserved (Itoh and Ornitz, 2004). A BLAST-search of the zebrafish cDNA and genomic DNA sequences with the amino acid sequence of human FGF22 identified a zebrafish amino acid sequence (207 amino acids) closely related to human FGF22 and mouse Fgf22 (supplementary material Fig. S1A). We isolated the full-length cDNA encoding the amino acid sequence from 24 hpf zebrafish embryo cDNA. Human *FGF22* is closely linked to the *BSG*, *HCN2*, and *POLRMT* genes on chromosome 19 at p13.3 (supplementary material Fig. S1B). Therefore, we have examined this gene's location in the zebrafish genome. The gene was also closely linked to the zebrafish *bsg*, *hcn2*, and *polrmt* genes on chromosome 22 (supplementary material Fig. S1B). Thus, this gene was identified as zebrafish *fgf22*.

### Expression pattern of *fgf22*

The temporal expression of *fgf22* during embryonic development was examined by RT-PCR. As shown in Fig. 1A, *fgf22* expression was first detected at low levels at 12 hpf. Subsequently, the expression gradually increased and was detected at least until 36 hpf.

We then investigated the spatiotemporal expression pattern of *fgf22* by whole mount in situ hybridization. At 14 and 16 hpf, *fgf22* was expressed near the posterior midbrain primordium (arrow), whereas *fgf22* expression was not detected in the most dorsal part (Fig. 1B,C,H,I,K,L). To examine the spatial expression pattern of *fgf22* in detail, the expression of *fgf22* at 14 hpf was compared with those of *wnt1*, *pax2.1* and *fgf8*, all of which are expressed persistently in the midbrain–hindbrain



**Fig. 1. Expression of *fgf22* in zebrafish embryos.** (A) Amplification of *fgf22* by RT-PCR at the indicated stages. The lower panel shows results for *eflα* as a control. (B–S) Expression pattern of *fgf22* (B–G, K–S) and *wnt1* (H–J) in zebrafish embryos at the indicated stages detected by whole-mount in situ hybridization. Embryos were double-labeled for *wnt1* (red) (N, Q), *pax2.1* (red) (O, R), or *fgf8* (red) (P, S). Lateral views with anterior to the left and dorsal to the top (B–D, G, N–S). Dorsal views with anterior to the top (F, H–M). Arrowheads and asterisks indicate the telencephalon and otic vesicles, respectively.

region (Lun and Brand, 1998). A comparison between *fgf22* and *wnt1* expression showed that the caudal boundary of *fgf22* expression was nearly identical to the caudal boundary of the *wnt1* expression domain (Fig. 1H, I, K, L, N). The caudal boundary of *fgf22* expression was located posteriorly to the rostral boundary of *pax2.1* expression and the posterior domain of

*fgf22* expression overlapped with the anterior domain of *pax2.1* expression (Fig. 1O). On the other hand, the *fgf8* expression domain was located at a distance from the *fgf22* expression domain, since *fgf8* was expressed in the posterior region contiguous to the *pax2.1* expression domain (Fig. 1P). These observations indicate that *fgf22* is expressed in both ventral and dorsal domains except most dorsal domain in the posterior midbrain and anterior MHB primordium. By 18 hpf, *fgf22* expression had intensified in the ventral domain in the posterior midbrain and anterior MHB (Fig. 1D, J, M, Q–S). At 24 hpf, *fgf22* expression was still detectable in the posterior midbrain (arrow), but no longer found in the anterior MHB (bracket) (Fig. 1E, F). The expression in the posterior midbrain (arrow) continued at least until 36 hpf (Fig. 1G). In addition, *fgf22* was expressed in the telencephalon and otic vesicles at 18 and 24 hpf, respectively (Fig. 1D, E). At 36 hpf, *fgf22* expression had intensified in both the telencephalon and the otic vesicles (Fig. 1G).

**Inhibition of *fgf22* functions results in defects in formation of the brain**

To examine the roles of *fgf22* in zebrafish development, we performed knockdown experiments with MOs. We injected two independent splice-site-targeted MOs (MO1 and MO2) for *fgf22* into 2-cell embryos and examined whether MOs could efficiently block the splicing of the *fgf22* mRNA precursor in zebrafish embryos (Fig. 2A). Although the wild-type cDNA was subjected to normal splicing, the amplified cDNA from *fgf22* MO1-injected embryos, which was shorter than the wild-type cDNA was subjected to abnormal splicing, resulting in a truncated translation product (Fig. 2B, C). In addition, the expression of mature *fgf22* mRNA was greatly decreased in *fgf22* MO2-injected embryos (Fig. 2B). These results indicate that both of the non-overlapping MOs effectively blocked the maturation of *fgf22* mRNA.

The *fgf22* morphants were morphologically defective in formation of the MHB constriction and exhibited a failure of the midbrain to evaginate laterally at 24 hpf (MO1, *n*=407/476 and MO2, *n*=79/99) (Fig. 2F, G, L, M). In addition, the *fgf22* morphants showed morphological abnormality in the forebrain at 24 hpf (Fig. 2F, G, L, M). On the other hand, the control MO-injected embryos developed normally during embryogenesis (*n*=25/25) (Fig. 2D, E). MOs might elicit undesirable off-target effects, which are rescued by co-knockdown of *tp53* (Gerety and Wilkinson, 2011). We examined whether the co-injection of *tp53* MO with *fgf22* MO1 could rescue the phenotype of *fgf22* MO1-injected embryos at 24 hpf. The co-injection of *tp53* MO with *fgf22* MO1 did not prevent the impaired neural development caused by *fgf22* MO1 (*n*=73/79) (Fig. 2H, I). Furthermore, the phenotype was also confirmed by RNA rescue experiments. The co-injection of *fgf22* RNA with *fgf22* MO1 rescued the defects in the brain caused by *fgf22* MO1 (*n*=37/51) (Fig. 2J, K). These results suggest that *fgf22* is required for the formation of the MHB constriction, and normal development of the forebrain and midbrain during neurogenesis.

Cell proliferation in the midbrain is reduced in *fgf22* morphants. In mice, Fgf signaling regulates cell proliferation and cell survival in the midbrain (Xu et al., 2000; Chi et al., 2003; Trokovic et al., 2003). Therefore, we examined whether a defect in cell proliferation and/or cell survival could account for the observed morphological abnormality in the midbrain of *fgf22* morphants. Phosphorylated histone H3 (pH3) was specifically detected in proliferating cells (Hendzel et al., 1997). We identified proliferating cells as pH3-positive



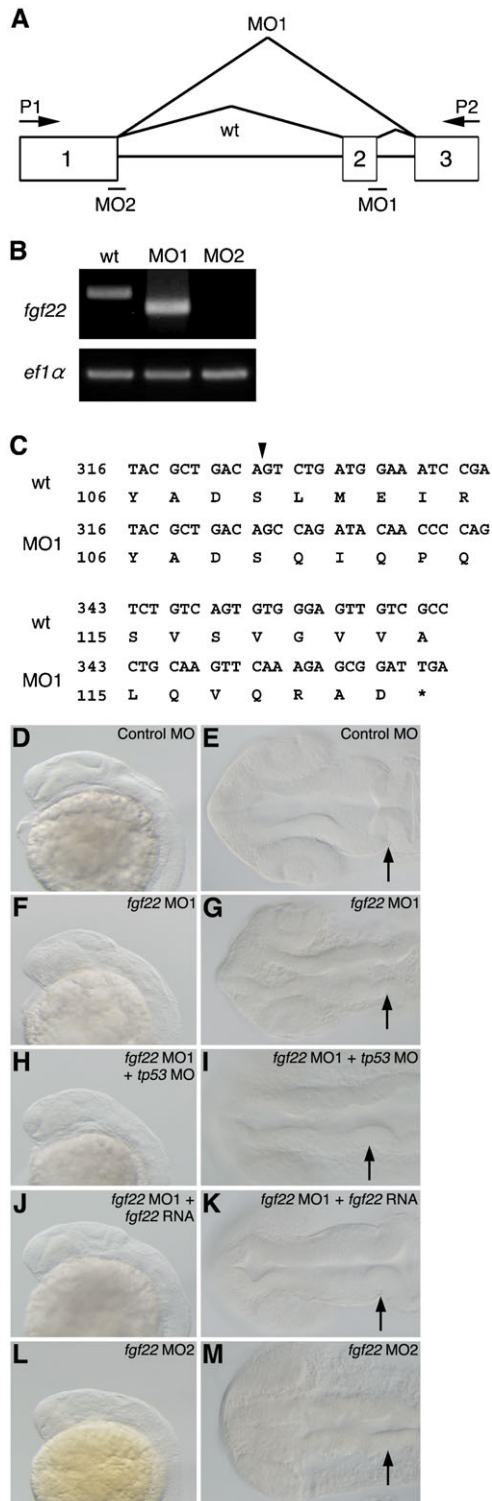
cells. The rate of pH3-positive cells in the midbrain of *fgf22* morphants was significantly decreased in comparison with that in wild-type embryos at 24 hpf (Fig. 3A,C,E). Conversely, the rate of pH3-positive cells in the midbrain was significantly increased in *fgf22* RNA-injected embryos (Fig. 3A,B,E). These results suggest that *fgf22* stimulates proliferation in the midbrain. Furthermore, *fgf22* morphants were assayed for apoptotic cells via TUNEL labeling at 24 hpf. The number

of apoptotic cells in the midbrain of *fgf22* morphants was slightly increased in comparison with that in the wild-type embryos ( $n=16/17$ ) (supplementary material Fig. S2A,B).

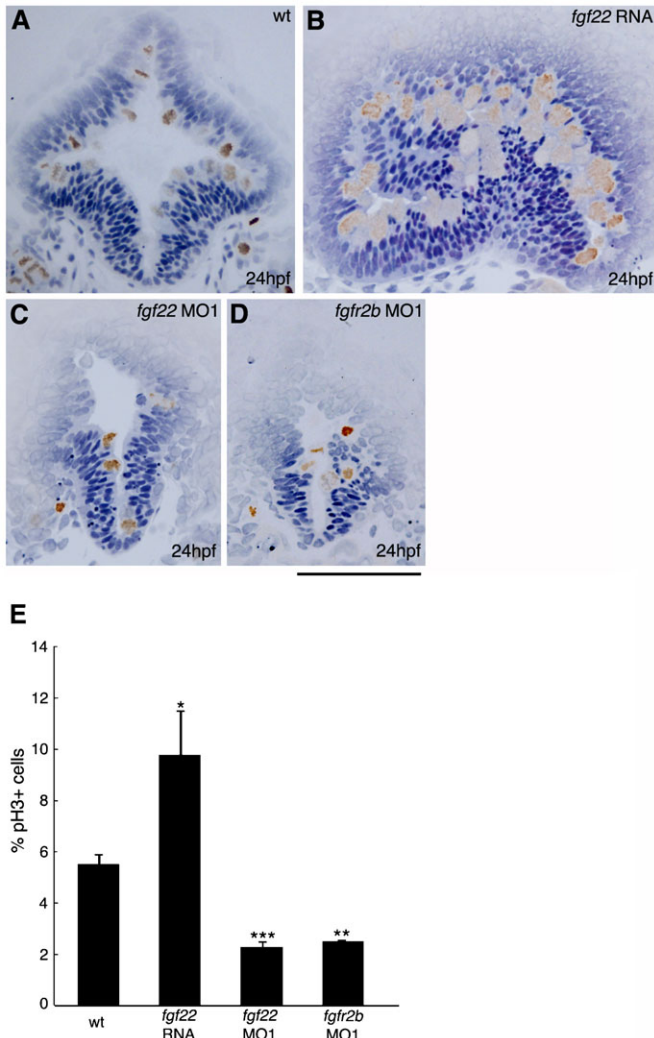
Expression of roof plate marker genes is expanded in the midbrain of *fgf22* morphants

The *fgf22* morphants showed morphological abnormality in the MHB constriction. Therefore, to investigate whether *fgf22* is implicated in MHB development, we examined the expression of genes related with MHB patterning in *fgf22* morphants at 24 hpf. In *fgf22* morphants, the expression of *pax2.1*, *her5*, and *eng2a* was detected in the MHB ( $n=27/27$ ,  $n=33/33$ , and  $n=23/23$ , respectively) (Fig. 4A–F). However, optical cross-sections showed that the expression of *pax2.1*, *her5*, and *eng2a* in the dorsal domain of the MHB was eliminated or reduced in *fgf22* morphants ( $n=19/27$ ,  $n=24/33$ , and  $n=17/23$ , respectively) (Fig. 4I–L; data not shown). On the other hand, the expression of *wnt1* was detected in both the dorsal and ventral domains of the MHB in *fgf22* morphants ( $n=24/24$ ) (Fig. 4G,H). These results indicate that loss of *fgf22* function disrupts normal specification of the dorsal domain in the MHB. *wnt1* is also expressed in the dorsal midline of the midbrain at 24 hpf (Fig. 5A). In *fgf22* morphants, the lateral expansion of *wnt1* expression was detected in the dorsal domain of the midbrain (MO1,  $n=24/24$  and MO2,  $n=21/21$ ) (Fig. 5A,B; supplementary material Fig. S3B). Furthermore, the expression of *msxb*, *lmx1b.2*, and *bmp5*, markers for the midbrain roof plate, in *fgf22* morphants was up-regulated in the dorsal midbrain and their expression domains were expanded at 24 hpf ( $n=31/32$ ,  $n=39/49$ ,  $n=15/16$ ), respectively (Fig. 5D,E,G,H,J,K). Conversely, *eng2a* expression was eliminated in the dorsal domain of the posterior midbrain in *fgf22* morphants ( $n=17/23$ ) (Fig. 4M,N). An analysis of transverse sections through the posterior midbrain showed that in *fgf22* morphants, the roof plate, which is characteristically thin and marks the dorsal midline of the neural tube, was similarly thin but much wider than normal (Fig. 4M,N). These results suggest that Fgf22 signaling suppresses the roof plate fate in the midbrain.

D/V pattern forms incorrectly in the midbrain of *fgf22* morphants As mesencephalic morphology was altered following *fgf22* knockdown, we investigated whether *fgf22* was involved in specification of the midbrain. *otx2* expressed in the midbrain is involved in midbrain patterning (Katahira et al., 2000). In *fgf22* morphants, *otx2* expression was down-regulated in the midbrain at 24 hpf ( $n=28/30$ ) (Fig. 6A,B). In particular, *otx2* expression in the most dorsal domain of the tectum was completely eliminated in *fgf22* morphants ( $n=28/30$ ) (Fig. 6D,E). In mice, *Otx2* is also an important player in the regulation of midbrain D/V patterning (Alexandre and Wassef, 2003). Therefore, we investigated



**Fig. 2. Inhibition of *fgf22* functions in zebrafish embryos.** (A) The coding region of *fgf22* is divided by two introns. Open boxes and black lines indicate exons and introns, respectively. MO indicates the target position of *fgf22* MO. (B) *fgf22* cDNA was amplified from wild-type or *fgf22* MO-injected embryonic cDNA by RT-PCR using P1 and P2 primers, the positions of which are indicated by arrows (A). *ef1α* cDNA was also amplified as a control. (C) The nucleotide sequences of *fgf22* cDNAs described above were determined. Numbers for the nucleotide sequence of the coding region and the amino acid sequence are shown. Arrowheads indicate splice-sites between exons one and two. (D–M) Lateral views (D,F,H,J,L) and dorsal views (E,G,I,K,M) of control MO-injected (D,E), *fgf22* MO1-injected (F,G), *fgf22* MO1- and *tp53* MO-injected (H,I), *fgf22* MO1- and *fgf22* RNA-injected (J,K), and *fgf22* MO2-injected (L,M) embryos at 24 hpf are shown.



**Fig. 3. Comparison of cell proliferation in the midbrain of embryos injected with *fgf22* RNA, *fgf22* MO1, or *fgfr2b* MO1.** (A–D) Wild-type embryos (A) and embryos injected with *fgf22* RNA (B), *fgf22* MO1 (C), or *fgfr2b* MO1 (D) were stained using an anti-pH3 antibody. Panels show representative transverse sections of the midbrain at 24 hpf. Scale bar: 100  $\mu$ m. (E) The percentage of proliferating cells labelled with anti-pH3 antibody in the midbrain of wild-type embryos and embryos injected with *fgf22* RNA, *fgf22* MO1, or *fgfr2b* MO1. Results are the mean  $\pm$  S.D. for three independent sections from three embryos. The statistical significance of differences in mean values was assessed with the Student's *t*-test. Asterisks indicate statistical significance compared with the wild type (\* $P$ <0.05; \*\* $P$ <0.01; \*\*\* $P$ <0.001).

whether *fgf22* is implicated in tectal fate specification. The expression of *meis2.2* and *pax7a* was reduced in the most dorsal domain and the ventral domain of the tectum in *fgf22* morphants at 24 hpf (MO1,  $n=14/14$ , MO1,  $n=15/15$ , and MO2,  $n=13/14$ ), respectively (Fig. 6G,H,I,K,P,Q; supplementary material Fig. S3D). Next, we investigated whether the reduction of tectal marker gene expression in *fgf22* morphants was accompanied by the alteration of ventral marker gene expression. In *fgf22* morphants, *nkx6.2* expression was partially expanded into the dorsal region of the midbrain at 24 hpf (MO1,  $n=21/23$  and MO2,  $n=16/17$ ) (Fig. 6M,N,R,S; supplementary material Fig. S3F). Taken together, these results demonstrate that Fgf22 is required for normal tectal and tegumental development.

### Inhibition of *fgfr2b* functions disrupts formation of both the dorsal and ventral midbrain

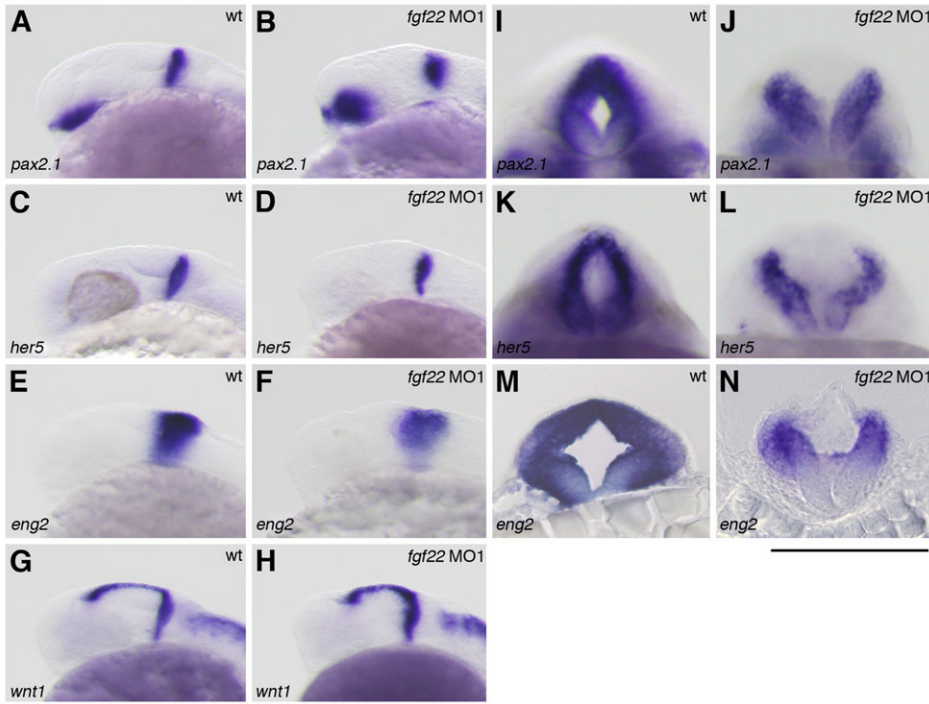
*Fgfr* genes contain an extracellular ligand-binding domain with three immunoglobulin-like domains (I, II and III), a transmembrane domain, and a split intercellular tyrosine kinase domain (Itoh and Ornitz, 2004). Among them, immunoglobulin-like domain III is involved in the determination of ligand-binding specificity and *Fgfr1-Fgfr3* encode two major versions of the domain (IIIb and IIIc) generated by alternative splicing (Itoh and Ornitz, 2004). Human FGF22 specifically bound to human FGFR2b *in vitro* and zebrafish *fgfr2* are expressed in the midbrain during somitogenesis (Zhang et al., 2006; Ota et al., 2010). These findings suggest *fgfr2b* to be involved in the roles of *fgf22* in the midbrain; therefore, we injected two splice-site-targeted MOs (MO1 and MO2) for *fgfr2b* into 2-cell embryos to investigate the role of *fgfr2b* in midbrain development. In embryos injected with *fgfr2b* MOs, the expression of mature *fgfr2b* mRNA was greatly decreased, whereas the expression of mature *fgfr2c* mRNA was unaffected (supplementary material Fig. S3A). As *fgfr2b* MOs could efficiently block the splicing of *fgfr2b* mRNA in embryos, we examined gene expression in the midbrain of *fgfr2b* morphants at 24 hpf. *wnt1* expression was expanded laterally (MO1,  $n=23/24$  and MO2,  $n=19/20$ ) (Fig. 5C; supplementary material Fig. S3C). The expression of *msxb*, *lmx1b.2*, and *bmp5* was also up-regulated in the dorsal midbrain ( $n=43/43$ ,  $n=26/28$ , and  $n=16/20$ , respectively) (Fig. 5F,I,L). On the other hand, *pax7a* expression was reduced in both the dorsal and ventral regions of the tectum (MO1,  $n=20/21$  and MO2,  $n=14/18$ ) (Fig. 6L; supplementary material Fig. S3E). The expression of *otx2* and *meis2.2* was also reduced in the tectum ( $n=22/22$  and  $n=20/22$ , respectively) (Fig. 6C,F,I). Conversely, *nkx6.2* expression in the tegmentum was expanded dorsally (MO1,  $n=11/11$  and MO2,  $n=13/15$ ) (Fig. 6O; supplementary material Fig. S3G). These results indicate that *fgfr2b* is involved in normal tectal and tegumental development.

Next, we examined proliferating cells in *fgfr2b* morphants at 24 hpf. The rate of pH3-positive cells in the midbrain was significantly decreased compared with that in wild-type embryos (Fig. 3D,E). These results suggest that *fgfr2b* is involved in cell proliferation. Thus, the phenotype of *fgfr2b* morphants was essentially similar to that of *fgf22* morphants.

### Phenotype of *fgf22* knockdown in the midbrain differs from that caused by inhibition of Hh signaling

Hh molecules produced in the floor plate function in D/V midbrain patterning. The misexpression of *Shh* in the midbrain transforms cell fate specification, from dorsal to ventral (Agarwala et al., 2001; Bayly et al., 2007). Conversely, no ventral cells remain and markers for dorsal cells are extended ventrally in the midbrain of *Shh* null mutants (Fedtsova and Turner, 2001; Fogel et al., 2008). As the alkaloid cyclopamine completely blocked Hh signaling at the level of Smoothened, which transduces hedgehog signals, in zebrafish (Taipale et al., 2000; Miyake et al., 2005), we analyzed the D/V midbrain patterning in embryos treated with cyclopamine. The embryos treated with cyclopamine from 5 hpf onwards showed a normal expression of *wnt1* in the dorsal midbrain at 24 hpf, whereas they showed a ventral expansion of *pax7a* expression and a loss of *nkx6.2* expression in the midbrain ( $n=13/16$ ,  $n=24/24$ , and  $n=22/22$ , respectively) (Fig. 7A,B,D,E,G,H). This result was consistent with that for *shh* null mutants, whereas the phenotype





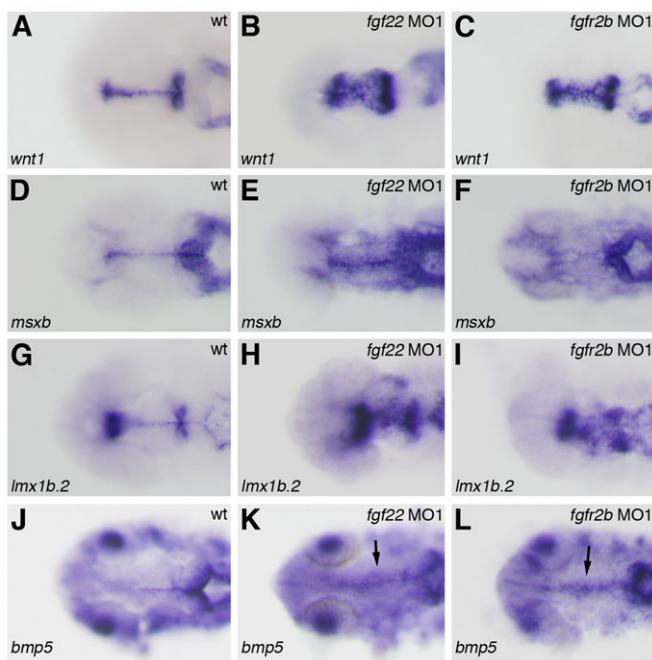
**Fig. 4. Gene expression in the MHB of the *fgf22* morphants.** (A–N) The expression of *pax2.1* (A,B,I,J), *her5* (C,D,K,L), *eng2* (E,F,M,N), and *wnt1* (G,H) in wild-type embryos (A,C,E,G,I,K,M) and *fgf22* morphants (B,D,F,H,J,L,N) at 24 hpf. A–H are lateral views, anterior to the left; I–L are optical cross-sections of the MHB; M,N are midbrain transverse sections. (I–N) Scale bar: 100 μm.

of *fgf22* morphants differed from that of the embryos treated with cyclopamine. Therefore, we examined whether *shh* expression was affected by inhibition of *fgf22*. *shh* expression was not affected in *fgf22* morphants at 24 hpf ( $n=33/33$ ) (Fig. 7J,K). Furthermore, we examined whether *fgf22* expression was responsive to Hh signaling. Surprisingly, *fgf22* expression was

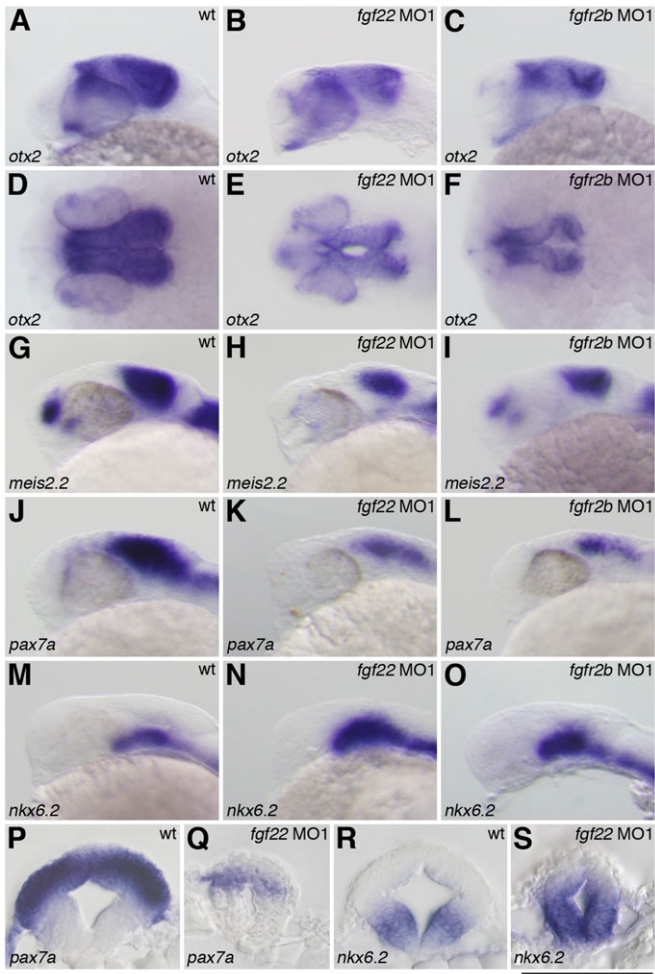
reduced in the posterior midbrain of the embryos treated with cyclopamine at 24 hpf ( $n=12/12$ ) (Fig. 7L,M). However, *fgf22* expression in the posterior midbrain was still detected in cyclopamine-treated embryos. Next, we investigated whether a dorsolateralization of the midbrain caused by blocking Hh signaling was affected by *fgf22* knockdown at 24 hpf. In the embryos injected with *fgf22* MO1 and treated with cyclopamine, *wnt1* expression was expanded laterally compared with that in the embryos treated with cyclopamine ( $n=18/19$ ) (Fig. 7C). This result suggests that *wnt1* is regulated by *fgf22* but not by Hh signaling in the midbrain. On the other hand, *pax7a* expression was strongly reduced in the embryos injected with *fgf22* MO1 and treated with cyclopamine compared with the embryos treated with cyclopamine ( $n=44/49$ ) (Fig. 7F). This result suggests that an expansion of *pax7a* expression in the midbrain caused by inhibition of Hh signaling is suppressed by inhibition of *fgf22*. A loss of *nkx6.2* expression in the midbrain caused by blocking Hh signaling was unaffected by *fgf22* knockdown ( $n=14/14$ ) (Fig. 7I). This result indicates that inhibition of *fgf22* does not rescue a loss of *nkx6.2* expression caused by inhibition of Hh signaling in the ventral midbrain.

***fgf22* expression in the midbrain is lost in *fgf3/fgf8* double morphant embryos**

Transplantation and ablation experiments in avian embryos have indicated that the isthmic organizer is involved in the positioning and development of the midbrain roof plate (Alexandre and Wassef, 2003). *Fgf8* induces the isthmic node and participates in the formation of the MHB and midbrain roof plate in avian embryos (Bally-Cuif and Wassef, 1994; Crossley et al., 1996; Alexandre et al., 2006). In zebrafish, *fgf3* and *fgf8* are expressed in the MHB (Reifers et al., 1998; Kwak et al., 2006). Therefore, we examined whether the expression of roof plate marker genes was affected by inhibition of Fgf3 and Fgf8 signaling. The embryos co-injected with *fgf3* MO and *fgf8* MO showed a lateral expansion of *wnt1*

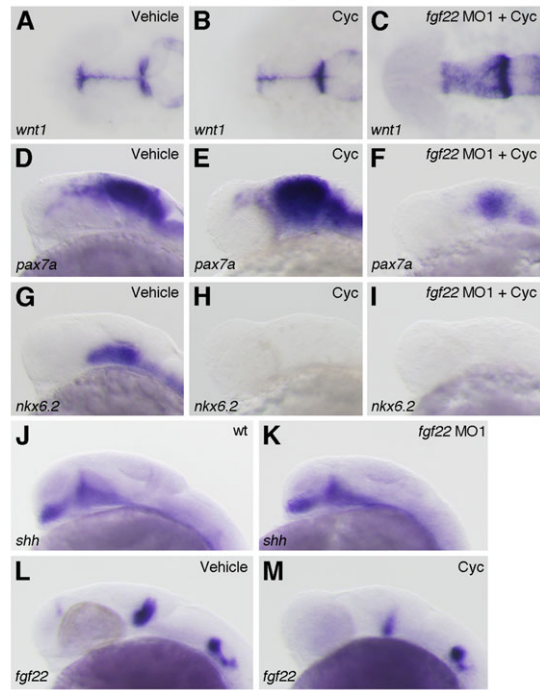


**Fig. 5. Gene expression in the midbrain roof plate of the *fgf22* and *fgfr2b* morphants.** The expression of *wnt1* (A–C), *msxb* (D–F), *lmx1b.2* (G–I), and *bmp5* (J–L) in wild-type embryos (A,D,G,J) and *fgf22* (B,E,H,K) and *fgfr2b* (C,F,I,L) morphants at 24 hpf.



**Fig. 6. Gene expression in the midbrain of the *fgf22* and *fgfr2b* morphants.** The expression of *otx2* (A–F), *meis2.2* (G–I), *pax7a* (J–L, P, Q) and *nkx6.2* (M–O, R, S) in wild-type embryos (A, D, G, J, M, P, R) and *fgf22* (B, E, H, K, N, Q, S) and *fgfr2b* (C, F, I, L, O) morphants at 24 hpf. A–C, G–O are lateral views, anterior to the left; D–F are dorsal views; P–S are midbrain transverse sections. (P–S) Scale bar: 100  $\mu$ m.

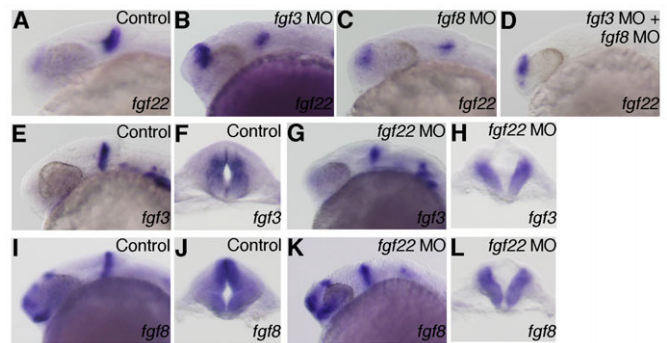
expression in the midbrain at 24 hpf ( $n=22/23$ ) (Fig. 9A). Furthermore, they showed a reduction of *pax7a* expression and ventral expansion of *nkx6.2* expression in the midbrain ( $n=36/45$  and  $n=15/20$ , respectively) (Fig. 9C, E). This phenotype is similar to that of *fgf22* morphants. In zebrafish, *fgf3* and *fgf8* are expressed in the MHB primordium at earlier stages than *fgf22* expression in the posterior midbrain primordium. Therefore, we examined whether *fgf22* expression in the midbrain was affected by inhibition of Fgf3 and Fgf8 signaling. Although *fgf22* expression was reduced in the posterior midbrain of the embryos injected with either *fgf3* MO or *fgf8* MO at 24 hpf, it was still detected ( $n=12/12$  and  $n=12/13$ , respectively) (Fig. 8A–C). On the other hand, *fgf22* expression was completely lost in the posterior midbrain of the embryos co-injected with *fgf3* MO and *fgf8* MO ( $n=19/19$ ) (Fig. 8D). In *fgf22* morphants, the expression of *fgf3* and *fgf8* was detected in the MHB ( $n=17/17$  and  $n=22/22$ , respectively) (Fig. 8E, G, I, K). The analysis of optical cross-sections showed that the expression of *fgf3* and *fgf8* was eliminated or reduced in the dorsal domain of the MHB in *fgf22* morphants ( $n=17/27$  and  $n=22/22$ , respectively)



**Fig. 7. Interactions between *fgf22* and Hh signaling in the midbrain.** (A–I) The expression of *wnt1* (A–C), *pax7a* (D–F) and *nkx6.2* (G–I) at 24 hpf in wild-type embryos treated with 0.95% ethanol (A, D, G) or cyclopamine (B, E, H) and *fgf22* morphants treated with cyclopamine (C, F, I). (J–M) The expression of *shh* (J, K) and *fgf22* (L, M) at 24 hpf in wild-type embryos (J), *fgf22* morphants (K), and wild-type embryos treated with 0.95% ethanol (L) or cyclopamine (M).

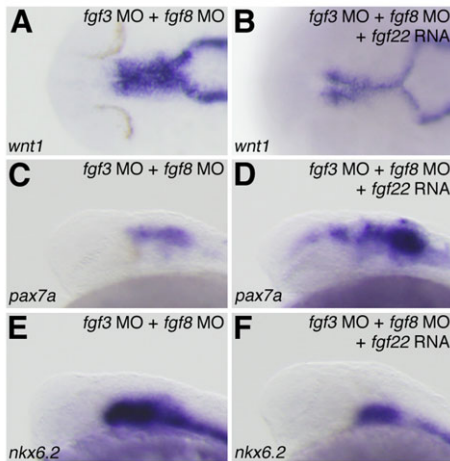
(Fig. 8F, H, J, L). This is possibly due to expansion of the roof plate, where *fgf3* and *fgf8* are not expressed. These results suggest that a combinatorial function of *fgf3* and *fgf8* is involved in the regulation of *fgf22* expression in the posterior midbrain but *fgf22* may not regulate *fgf3* and *fgf8* expression in the MHB.

Next, to investigate whether *fgf3*- and *fgf8*-mediated loss of *fgf22* function leads to defects in dorsal midbrain specification, we injected *fgf3/fgf8* double morphants with *fgf22* RNA. The injection depressed an expansion of *wnt1* and *nkx6.2* expression



**Fig. 8. Interactions between *fgf3*, *fgf8* and *fgf22*.** (A–D) The expression of *fgf22* at 24 hpf in embryos injected with control MO (A), *fgf3* MO (B), *fgf8* MO (C), and *fgf3* MO and *fgf8* MO (D). (E–L) The expression of *fgf3* (E–H) and *fgf8* (I–L) at 24 hpf in embryos injected with control MO (E, F, I, J) and *fgf22* MO (G, H, K, L). A–D, E, G, I, K are lateral views, anterior to the left; F, H, J, L are optical cross-sections of the MHB.





**Fig. 9. Rescue of midbrain region-specific marker loss in *fgf3/fgf8* morphants by *fgf22* RNA.** The expression of *wnt1* (A,B), *pax7a* (C,D), and *nkx6.2* (E,F) at 24 hpf in *fgf3/fgf8* morphants (A,C,E) and *fgf22* RNA-injected *fgf3/fgf8* morphants (B,D,F).

in the dorsal midbrain caused by co-injection of *fgf3* MO and *fgf8* MO ( $n=24/26$  and  $n=30/33$ , respectively) (Fig. 9B,F). Furthermore, *pax7a* expression was up-regulated in the dorsal midbrain of *fgf3/fgf8* double morphants injected with *fgf22* RNA ( $n=25/32$ ) (Fig. 9D). These results indicate that *fgf22* partially rescues the phenotype caused by inhibition of *fgf3* and *fgf8* function in the specification of the dorsal midbrain.

#### Blocking proliferation does not contribute to specification of the dorsal midbrain

As the decreased proliferation of tectal precursors might contribute to the reduction in the *pax7a* expression domain of the dorsal midbrain, we addressed whether decreases in cellular proliferation can secondarily cause patterning defects. To block proliferation, wild-type embryos were treated with hydroxyurea and aphidicholin (HUA), which have been used previously in zebrafish to reduce proliferation (Ikegami et al., 1999; Lyons et al., 2005). We applied HUA to embryos at 8 hpf, and analyzed midbrain development at 24 hpf. HUA treatment effectively inhibited proliferation, as evidenced by a reduction in the number of pH3-positive cells in the midbrain ( $n=7/7$ ) (supplementary material Fig. S4A,B). However, the domain of *pax7a* expression appeared relatively normal ( $n=22/23$ ) (supplementary material Fig. S4E,F). In addition, we observed no shift of *wnt1* and *nkx6.2* into the dorsal domain ( $n=23/23$  and  $n=26/26$ , respectively) (supplementary material Fig. S4C,D,G,H). These results argue that the midbrain patterning defects that arise when Fgf signaling is disrupted are not due to decreases in localized proliferation.

#### Discussion

##### *fgf22* controls cell proliferation in the midbrain

Fgf signaling regulates the proliferation and differentiation of specific neuronal cell types in the midbrain (Ye et al., 1998; Xu et al., 2000; Trokovic et al., 2005). Among the *fgf* family, *fgf22* showed a unique expression pattern in the midbrain and MHB primordia. *fgf22* morphants showed a decrease in tectal volume. *fgf22* knockdown significantly inhibited cell proliferation in the midbrain. However, the knockdown did not strongly stimulate

apoptosis in the midbrain. In addition, overexpression of *fgf22* resulted increased cell proliferation in the midbrain. These results indicate that the reduction of tectum volume was not due to apoptosis rather due to the decreased cell proliferation in *fgf22* morphants. *fgfr2b* knockdown also resulted in decreased cell proliferation in the midbrain and *fgfr2b* morphants showed very similar morphological defects to those obtained by *fgf22* knockdown. Thus, it is suggested that Fgf22 signaling is mediated through Fgfr2b during cell proliferation in the midbrain.

##### *fgf22* is involved in formation of the roof plate

Roof plate cells are induced to form by Bmp signals from the epidermal ectoderm and develop at the dorsal midline of the neural tube (Liem et al., 1995). Members of the Msx family have been implicated as downstream targets of Bmps and are induced to express in regions where Bmp signaling is active (Furuta et al., 1997; Graham et al., 1994; Liem et al., 1995; Shimeld et al., 1996; Timmer et al., 2002). Bmp signaling can be inhibited by Fgf signaling in the forebrain and midbrain (Storm et al., 2003; Alexandre et al., 2006). In zebrafish, *bmp5* and *msxb* are expressed in the midbrain roof plate (Miyake et al., 2012) and the expression of *bmp5* and *msxb* was increased in *fgf22* morphants. This suggests that Fgf22 regulates Bmp signaling in the midbrain. Overexpression of *Msx1* induces the ectopic expression of *Lmx1* and *Wnt1* (Liu et al., 2004). *Lmx1b* is sufficient to form a functional roof plate in the hindbrain and spinal cord (Chizhikov and Millen, 2004; Mishima et al., 2009). The increased expression of *wnt1* and *lmx1b.2* in the midbrain of *fgf22* morphants may be due to an expansion of *msxb* expression. Therefore, Fgf22 may function to suppress the mediolateral extent of Bmp signaling from the center of the roof plate in the midbrain. On the other hand, loss of Fgf22 function led to a loss of MHB markers in the dorsal MHB region. This result suggests Fgf22 to be involved in the specification of the dorsal MHB region. However, the defect in the dorsal domain of the MHB might be due to the lateral expansion of the midbrain roof plate in *fgf22* morphants. Furthermore, *fgfr2b* knockdown resulted in the expanded expression of roof plate markers and *fgfr2b* morphants showed very similar dorsal patterning defects to those observed after *fgf22* knockdown. Thus, it is suggested that Fgf22 signaling suppresses the roof plate fate in the midbrain and it is mediated through Fgfr2b.

##### *fgf22* is required for specification of the tectum

Otx2 is essential for the formation of all forebrain- and midbrain-derived structures (Acampora et al., 1995). *Meis2* is both necessary and sufficient for tectal fate specification (Agoston and Schulte, 2009). *Meis2* acts downstream of Otx2 and is a direct partner of Otx2 in the tectum (Agoston and Schulte, 2009). In *fgf22* morphants, the expression of *otx2* and *meis2.2* was reduced in the midbrain. In addition, *pax7a* expression in the tectum was reduced in *fgf22* morphants. *fgf22* knockdown resulted in decreased proliferation and *fgf22* morphants showed a decrease in tectal volume. However, decreased proliferation is not sufficient to cause patterning defects in the midbrain, as *pax7a* expression in the alar plate was not reduced in the midbrain in embryos treated with HUA in spite of decreased proliferation in this domain. Thus, reduced proliferation is not a major mechanism contributing to the reduction of tectal cell fate in *fgf22* morphants. These results indicate that *fgf22* is required for the specification of the tectum.



The roof plate is an important signaling center that controls dorsal CNS patterning and specification through secretion of the Bmp and Wnt signaling molecules. In *fgf22* morphants, dorsal *pax7a* expression was reduced and the roof plate markers shifted into the domain where *pax7a* expression was absent. Because *pax7a* is not expressed in the roof plate, the decreased expression of *pax7a* in the tectum might cause the expanded expression of the roof plate markers in *fgf22* morphants. Ventral *nkx6.2* expression also shifted into the dorsal domain in the midbrain of *fgf22* morphants, whereas *fgf22* knockdown did not induce the expression of *nkx6.2* in embryos with blocked Hh signaling. On the other hand, *fgf22* knockdown strongly suppressed the up-regulation of *pax7a* expression caused by blocking Hh signaling. These results indicate that *fgf22* is not involved in specification of the tegmentum and the increased expression of *nkx6.2* in *fgf22* morphants may be due to a reduction of *pax7a* expression. Thus, it is suggested that *fgf22* is involved in specification of the tectum by controlling *pax7a* expression. Furthermore, *fgfr2b* knockdown resulted in the decreased expression of *pax7a* and the increased expression of *nkx6.2*. The loss of the dorsal midbrain in the morphants might secondarily induce expansion of the most dorsal tissues in the midbrain. Therefore, Fgf22 signaling is suggested to be mediated through Fgfr2b in the specification of the tectum.

*fgf3* and *fgf8* are required for *fgf22* expression in the posterior midbrain

Hh signaling is involved in D/V patterning of the midbrain. Cross talk between Fgf and Hh signaling is critical for brain development (Brewster et al., 2000). *fgf22* expression in the posterior midbrain was reduced in embryos with blocked Hh signaling, whereas *shh* expression was unaffected in *fgf22* morphants. However, we speculate that *fgf22* expression in the posterior midbrain is reduced by a secondary effect of dorsalization of the midbrain in embryos with blocked Hh signaling, because the phenotype of *fgf22* morphants was opposite to that of embryos with blocked Hh signaling. Thus, the function of *fgf22* differed from that of Hh signaling in the development of the midbrain roof plate and the specification of the tectum.

The isthmic organizer is implicated in the formation of the caudal roof plate in the midbrain and is crucial for the growth and patterning of the midbrain (Martínez, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001; Alexandre and Wassef, 2003). When the isthmic tissue is ablated, the roof plate of the caudal midbrain fails to develop (Alexandre and Wassef, 2005). *Fgf8* is a key component of the isthmic organizer and Fgf8 bead implantation experiments have demonstrated that isthmic organizer signals, in particular Fgf8, are involved in the positioning and differentiation of the midbrain roof plate (Alexandre and Wassef, 2005). In zebrafish, *fgf3* and *fgf8* are expressed in the MHB (Reifers et al., 1998; Kwak et al., 2006) and at earlier stages than *fgf22*. In the *fgf3/fgf8* double morphant embryos, *fgf22* expression was completely lost in the posterior midbrain. This result indicates that *fgf22* expression in the posterior midbrain is regulated by Fgf3 and Fgf8 signaling from the MHB territory. The phenotype of the *fgf3/fgf8* double morphant embryos was similar to that of *fgf22* morphants. Furthermore, injection of *fgf22* RNA into *fgf3/fgf8* double morphant embryos rescued the reduction of *pax7a* expression and the expansion of *wnt1* and *nkx6.2* expression caused by co-injection of *fgf3* MO and *fgf8* MO. Thus, *fgf22* is implicated in

the development of the midbrain roof plate and the specification of the tectum as a downstream factor of Fgf3 and Fgf8 signaling.

The present study indicates that Fgf22 is involved in cell proliferation, roof plate formation, and tectum specification through Fgfr2b in the zebrafish midbrain. Furthermore, *fgf22* is implicated in midbrain development as a factor downstream of *fgf3* and *fgf8* expressed in the MHB but not of *Hh* expressed in the floor plate. The present findings should provide new insights into roles of Fgf signaling in midbrain development in zebrafish. However, phenotypes of *fgf22* knockdown in zebrafish differ from those of *Fgf22*-deficient mice. In *Fgf22*-deficient mice, the differentiation of excitatory nerve terminals on dendrites of CA3 pyramidal neurons in the hippocampus and the development of retinal terminals in the dorsal lateral geniculate nucleus are impaired (Terauchi et al., 2010; Singh et al., 2012). As no distinct expression of *Fgf22* was observed in mouse embryos (Yaylaoglu et al., 2005), the different phenotypes may be due to different expression patterns in zebrafish and mice. In mice, other Fgfs expressed in the midbrain may be involved in cell proliferation, roof plate formation, and tectum specification in midbrain. This will be addressed in a future study.

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### Competing Interests

The authors have no competing interests to declare.

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