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

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Biology Open 2, 515–524
doi: 10.1242/bio.20134226
Received 24th January 2013
Accepted 1st March 2013

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). Fgfr2 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 Fgf22b 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 isolated cDNA is AB254028.

The positions of zebrafish fgf22, bg, hcn2, and polrmt on chromosome 22 were determined by the hours post fertilization (hpf) and by morphological features, as described by Kimmel et al. (Kimmel et al., 1995).

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), parx1 (Krauss et al., 1991), otx2 (Mori et al., 1994), eng2 (Ekker et al., 1992), her5 (Müller et al., 1996), Fgf9 (Reifers et al., 1998), Fgf10 (Phillips et al., 2001), nkx6.2 (Günner and Karlström, 2007), parx7a (Seo et al., 1998), lmx1b2 (Elsen et al., 2008), bmp1 (Holzschuh et al., 2005), metc2a (Waskiewicz et al., 2001), and mshx (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/iron 2 splice-blocking MO1, 5′-ATGCGATGTACCTACCGATCCGAAAG-3′; fgf22 exon 1/iron 2 splice-blocking MO2, 5′-GACCTGTGATCTACCTCCTCCTCACTCCTAC-3′; fgf22 exon 7/iron 7 splice-blocking MO1, 5′-CTCCGTGGGTCACTTATGCTTACATACG-3′; fgf22 exon 7/iron 7 splice-blocking MO2, 5′-GCATGATCTCTGGGTGAATTCG-3′; fgf22 exon 7/iron 7 splice-blocking MO3, 5′-CCCGTCTGGTTTCTCTGTGT-3′; and universal control MO, 5′-GCCTTATCTACTACG-3′. The positions of zebrafish Fgf22 cDNA and genomic DNA sequences with the amino acid sequence of human FGF22 were isolated by polymerase chain reaction (PCR) with zebrafish embryonic cDNA as a template. The GenBank accession number for the isolated cDNA is AB254028.

To determine the efficacy of MOs, RNA was isolated from wild-type, fgf22 MO1, fgf22 MO2, fgf22 MO1+MO2, and fgf22 MO2-injected embryos. cDNA was amplified from the RNA by RT-PCR using the above primers and the following primers (5′ primer/3′ primer): fgf22 ex2, 5′-GAGCTCGGCCAATACACAGCT-3′/5′-CTGGAGGATTACCGTCTCG-3′ (176 bp fragment) and fgf22 ex5, 5′-GACGACGCGTTGGAACACTAGCT-3′/5′-CTGAGGAATATCCTCGTCG3′ (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 into 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 homologous 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 FGFR22 and mouse Fgf22 (supplementary material Fig. S1A). We isolated the full-length cDNA encoding the amino acid sequence from 24 hp of zebrafish embryonic 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 bg, 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, parx2.1, and fgf8, all of which are expressed persistently in the midbrain–hindbrain
fgf22 expression overlapped with the anterior domain of pax2.1 expression (Fig. 1O). On the other hand, the fgf22 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 msx3, 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 characterized by a 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 dorsal 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

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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 embryoic 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.
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 intracellular 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 (*IIb* and *IIc*) generated by alternative splicing (Itoh and Ornitz, 2004). Human *FGF22* specifically bound to human FGFR2b in *vitro* and zebrafish *fgf2* 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 *fgf2* in the midbrain; therefore, we injected two splice-site-targeted MOs (MO1 and MO2) for *fgf2b* into 2-cell embryos to investigate the role of *fgfr2b* in midbrain development. In embryos injected with *fgfr2b* MOs, the expression of *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,LL). 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, *nkk6.2* 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 tegmental 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 (Fedtscova 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 *nkk6.2* 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

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,J,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, *nkk6.2* 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 tegmental development.
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. 7I,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 dorsalization 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 *nrx6.2* 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 *nrx6.2* 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*...
expression in the midbrain at 24 hpf ($n=22/23$) (Fig. 9A). Furthermore, they showed a reduction of $pax7a$ expression and $nkx6.2$ 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. 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. 8F,H,J,L).
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 nkkx6.2 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 an 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 (Li 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; Li 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 nkd6.2 expression also shifted into the dorsal domain in the midbrain of fgf22 morphants, whereas fgf22 knockdown did not induce the expression of nkd6.2 in embryos with blocked Hh signaling. On the other hand, fgf22 knockout 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 nkd6.2 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 knockout resulted in the decreased expression of pax7a and the increased expression of nkd6.2. 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 isthmic organizer signals, in particular Fgf8, are involved in the dorsoventral patterning in the mid/hindbrain by generating roof plate structures. Development 130, 5331-5338.


Development 130, 5331-5338.

Acknowledgements

We wish to thank Y. Nakagawa and T. Mido for technical assistance. This work was in part supported by Grant-in-Aid for Exploratory Research No. 23659035 (to N.I.) and for Young Scientists (B) No. 21790075 (to A.M.) from the Japan Society for the Promotion of Science.

Competing Interests

The authors have no competing interests to declare.

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Meis2 competes with the Groucho co-repressor Tle4 for binding to Otx2 and specifies tectal fate without induction of a secondary midbrain-hindbrain boundary organizer. Development 136, 3311-3322.


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