Fgf16 is required for specification of GABAergic neurons and oligodendrocytes in the zebrafish forebrain.
Fgf16 Is Required for Specification of GABAergic Neurons and Oligodendrocytes in the Zebrafish Forebrain

Ayumi Miyake*, Tatsuya Chitose, Eriko Kamei, Atsuko Murakami, Yoshiaki Nakayama, Morichika Konishi, Nobuyuki Itoh
Department of Genetic Biochemistry, Kyoto University Graduate School of Pharmaceutical Sciences, Sakyo, Kyoto, Japan

Abstract
Fibroblast growth factor (Fgf) signaling plays crucial roles in various developmental processes including those in the brain. We examined the role of Fgf16 in the formation of the zebrafish brain. The knockdown of fgf16 decreased cell proliferation in the forebrain and midbrain. fgf16 was also essential for development of the ventral telencephalon and diencephalon, whereas fgf16 was not required for dorsoventral patterning in the midbrain. fgf16 was additionally required for the specification and differentiation of γ-aminobutyric acid (GABA)ergic interneurons and oligodendrocytes, but not for those of glutamatergic neurons in the forebrain. Cross talk between Fgf and Hedgehog (Hh) signaling was critical for the specification of GABAergic interneurons and oligodendrocytes. The expression of fgf16 in the forebrain was down-regulated by the inhibition of Hh and Fgf19 signaling, but not by that of Fgf3/Fgf8 signaling. The Fgf16 morphant phenotype was similar to that of the fga19 morphant and embryos blocked Hh signaling. The results of the present study indicate that Fgf16 signaling, which is regulated by the downstream pathways of Hh-Fgf19 in the forebrain, is involved in forebrain development.

Introduction
The forebrain becomes regionally subdivided into the telencephalon and diencephalon during early embryonic brain development in vertebrates. The telencephalon is further subdivided into the rostrally positioned subpallial (ventral) telencephalon and more caudally located pallial (dorsal) telencephalon. The diencephalon is comprised of the hypothalamus, zona limitans intrathalamica (ZLI), ventral thalamus, dorsal thalamus, and pretectum [1]. The regional specification, growth, and differentiation of telencephalic and diencephalic subdivisions are controlled by interactions between secreted signaling molecules. The dorsal region of the telencephalon coordinates growth and patterning via Bone morphogenetic proteins (Bmps) and Wnts [2]. On the other hand, Hedgehog (Hh) signaling is known to be critical for ventral patterning in the forebrain and midbrain [3–5]. Fibroblast growth factor (Fgf) signaling has also been implicated in dorsoventral patterning and the regulation of cell proliferation and differentiation in various regions during brain development [1,6–9].

Fgfs comprise a large family of at least 22 members in vertebrates [10]. Of these, Fgf8 specifies rostral telencephalic fate and represses caudal telencephalic fate in mice and zebrafish [11–16]. Furthermore, the ectopic expression of fga3 in zebrafish affects the expression of genes that have been implicated in the development of the forebrain [17] and the knockdown of both fga3 and fga8 functions revealed that fga3 and fga8 possessed a unique and combinatorial function in regional patterning of the forebrain and hindbrain [7,18–20]. In contrast, an analysis of Fgf15 knockout mice demonstrated that Fgf15 repressed rostral telencephalic fate [21]. On the other hand, the function of fga19, which is the Fgf15 orthologue in zebrafish, is known to be essential for development of the ventral region of the telencephalon and diencephalon in zebrafish [8].

Fgf16, which was originally identified in the rat heart, is predominantly expressed in the heart at adult stages [22,23]. Fgf16 is expressed in the heart, inner ear and brown adipose tissue during embryonic development in mammals [22,24–27]. Three lines of Fgf16 knockout mice have been reported and their phenotypes may potentially be affected by genetic backgrounds. Fgf16 knockout mice on a C57BL/6 background exhibited a decrease in the proliferation of embryonic cardiomyocytes and pathophysiological roles in the postnatal heart, whereas the cardiac phenotype of Fgf16 knockout mice on a 129/B6 background has not yet been examined [23,27,28]. These two lines are viable, whereas Fgf16 knockout mice on a Black Swiss background died at approximately E11.5 [26]. Fgf16 is expressed in zebrafish in the pectoral fin bud and forebrain in addition to the olfactory vesicle [29]. An analysis of fga16 knockdown zebrafish embryos indicated that fga16 is an apical ectodermal ridge (AER) factor that is crucial for pectoral fin bud outgrowth [29]. In addition, fga16 morphants display morphological abnormalities in
the brain. However, these abnormalities have not yet been elucidated in detail.

In the present study, we examined the roles of fgf16 during brain development in zebrafish. Our results demonstrated that fgf16 was critical for cell proliferation in the forebrain and midbrain. fgf16 was also critical for development of the ventral region of the telencephalon and diencephalon, and was implicated in the specification of γ-aminobutyric acid (GABAergic) interneurons and oligodendrocytes in the telencephalon and diencephalon. On the other hand, fgf16 was not implicated in the specification of tectal and tegmental fates. fgf16 have also been shown to be involved in the specification of GABAergic interneurons and oligodendrocytes in the ventral region of the forebrain [8]. Thus, we also examined the crosstalk between fgf16 and fgf3, fgf8, and fgf19 in the forebrain.

Materials and Methods

Fish maintenance

Zebrafish (Danio rerio) were maintained, according to The Zebrafish Book [30]. 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 morphological features, as described by Kimmel et al. [31]. All animal studies were conducted according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Kyoto University Graduate School of Pharmaceutical Sciences (KUGSPS). The animal protocol was approved by the IACUC of KUGSPS; the approved protocol number was 2014-54.

Whole mount in situ hybridization

Digoxigenin-labeled RNA probes were synthesized by in vitro transcription using T7 or SP6 RNA polymerase. The fgf16 probe was synthesized using the full-length cDNA-containing plasmid. The other probes used were zebrafish emx1 [32], tbr1 [33], dlx2 [34], sbb [35], pax6a [36], ngn1 [37], isl1 [38], otx2 [39], nks6.2 [40], pax7a [41], fgf8 [12], gdf1 [42], slc17a6a [43], olg2 [44] and mlp [44]. Whole mount in situ hybridization was performed as previously described [45].

Morpholino and mRNA injection

Morpholino oligonucleotides (MOs) were synthesized by GeneTools, LLC (Corvallis, OR). MOs were diluted in Danieau buffer [46]. Universal control MO, fgf3 MO, fgf8 MO, fgf16 MO, and fgf19 MO have been reported previously [8,29,47]. fgf16 MO (5 ng) or universal control MO (5 ng) was injected into the two-cell embryos of zebrafish. fgf3 MO (10 μg/μl) and fgf8 MO (20 μg/μl) were injected at a volume of 0.15-0.25 nl into the two-cell embryos. fgf19 MO was injected at 10 μg/μl into the four central blastomeres of 16-cell embryos.

To construct fgf16, full-length fgf16 cDNA was amplified by PCR and inserted into the vector pCS2+[48]. Capped fgf16 mRNA was made by in vitro transcription using SP6 polymerase (mMESSAGE mACHINE; Ambion). mRNA was diluted to 0.5 ng/μl with distilled water and injected at a volume of 0.5 nl into 2-cell embryos.

H3P antibody staining and TUNEL labeling

Proliferating and apoptotic cells were detected using a rabbit polyclonal anti-phosphorylated histone H3 (H3P) (Upstate Biotechnology) antibody and the DeadEndTM colorimetric detection kit (Promega), respectively [8]. For cell counts, the stained embryos were embedded in Technovit 7100 ( Heraeus Kulzer, Wehrheim) and cut into 4-μm serial sections. These sections were then counterstained with hematoxylin.

Immunohistochemistry

Whole mount immunostaining was performed as described previously [49]. The following primary antibodies were used: rabbit anti-GABA (1:1000; Sigma) [50] and mouse anti-APC (1:30; Calbiochem) [51–53]. We used Alexa Fluor 488 goat anti-rabbit or anti-mouse IgG (1:200; Molecular Probes) for fluorescent detection.

Cyclopamine treatments

Cyclopamine (Toronto Chemical) [54] was dissolved at 10 mM in 95% ethanol. Embryos, which were 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.

Results

Inhibition of fgf16 functions resulted in defects in brain formation

We previously showed that zebrafish fgf16 was expressed in the pectoral fin bud and also that the knockdown of fgf16 function resulted in the absence of fin bud outgrowth at 5 days post-fertilization (dpf) [29]. In addition, the brain structures of fgf16 morphants exhibited abnormalities at 5 dpf [29]. fgf16 morphants were morphologically distinguishable from the wild type at 24 hours post-fertilization (hpf). fgf16 morphants showed morphological abnormalities in the forebrain at 24 hpf (Fig. 1B). Furthermore, fgf16 morphants were morphologically defective in the formation of midbrain-hindbrain boundary (MHB) constriction and exhibited a failure to evaginate laterally in the midbrain at 24 hpf (Fig. 1B). The gross morphological phenotypes obtained by an injection of either fgf16 MO1 or fgf16 MO2 were similar to each other (MO1, n = 78/89 and MO2, n = 79/112). On the other hand, control MO-injected embryos developed normally.
normally during embryogenesis [9]. Furthermore, the phenotype was confirmed by RNA rescue experiments. The co-injection of fgf16 RNA with fgf16 MO1 rescued the brain defects caused by fgf16 MO1 (n = 10/13) (Fig. 1C). These results suggested that fgf16 may be required for normal development in the forebrain and midbrain, and the formation of MHB constriction during neurogenesis.

Expression pattern of fgf16 in the brain

ggf16 is expressed in the brains of zebrafish embryos during 18–36 hpf [29]. However, the expression profile of fgf16 has not yet been examined in detail in the brain during neural development. We here examined the spatiotemporal expression pattern of fgf16 in the zebrafish embryonic brain in detail using whole mount in situ hybridization. The expression of fgf16 was first detected in the most ventral part of the anterior telencephalon primordium at 14 hpf (Fig. 2A). By 18 hpf, its expression had intensified in the telencephalon and the expression domain had expanded into the dorsal region (Fig. 2B). In addition, fgf16 was expressed in the diencephalon and midbrain at low levels (Fig. 2B). Its expression was maintained in the forebrain and the midbrain at 24 hpf (Fig. 2C). In addition, the strong expression of fgf16 was detected in the epiphysis and pituitary gland at 24 hpf (Fig. 2C). The expression of fgf16 had intensified in the diencephalon and ventral region of the midbrain at 36 hpf and its expression in the telencephalon was markedly decreased (Fig. 2D).

fgf16 was required for cell proliferation in the forebrain and midbrain

Fgf signaling has been shown to regulate cell proliferation and cell survival in the brains of mice and zebrafish [8,55,56]. fgf16 is also required for cell proliferation in the mesenchyme of fin buds [29]. Therefore, the morphological abnormalities observed in the forebrain and midbrain of fgf16 morphants at 24 hpf may have been due to a defect in cell proliferation and/or cell survival in these regions. To examine this, we compared the number of mitotic cells in wild-type embryos and fgf16 morphants. Phosphorylated histone H3 (pH3) was specifically detected in the mitotic cells in mitotic phase (M-phase) [57]. We identified proliferating cells as pH3-positive cells. The rate of pH3-positive cells in the forebrain of fgf16 morphants was significantly lower than that in wild-type embryos at 24 hpf (Fig. 3A–C). In addition, the rate of pH3-positive cells in the midbrain was significantly decreased in fgf16 morphants (Fig. 3A, B, D). These results suggested that fgf16 may promote cell proliferation in the forebrain and midbrain.

fgf16 was required for the development of the subpallial telencephalon and ventral thalamus

fgf3, fgf8, and fgf19 have been implicated in patterning events in the zebrafish forebrain [7,8,18–20]. Therefore, we investigated whether Fgf16 was also involved in the regionalization of the forebrain. The expression of telencephalon marker genes was analyzed in fgf16 morphants at 24 hpf. The expression of emx1, which is normally detected in the pallial domain of the telencephalon, was observed in the entire region of the telencephalon in fgf16 morphants (n = 28/32) (Fig. 4A, B). Furthermore, the expression of tbr1, which normally occurs in the pallial telencephalon, was also detected in the entire telencephalon in fgf16 morphants (n = 15/16) (Fig. 4C, D). In contrast to the expression of emx1 and tbr1, that of dlx2, which is normally detected in the ventral region of the telencephalon, was reduced in fgf16 morphants (n = 27/31) (Fig. 4E, F). On the other hand, the expression of pax6a, which is normally detected in the telenceph-
alon was unaffected in fgf16 morphants (n = 21/21) (Fig. 4G, H). The ectopic expression of otx2 was detected in the ventral region of the telencephalon in fgf16 morphants at 24 hpf (n = 13/13) (Fig. 5C, D). In contrast, all control embryos showed normal expression patterns for these genes (data not shown). These results indicated that fgf16 was required for the development of the subpallial telencephalon.

We also determined whether the inhibition of fgf16 affected diencephalic specification. In addition to the ventral telencephalon, dlx2 is normally expressed in the ventral thalamus. The expression of dlx2 in the ventral thalamus was reduced in fgf16 morphants at 24 hpf (n = 27/31) (Fig. 4E, F). On the other hand, the expression of pax6a and otx2 in the diencephalon was unaffected in fgf16 morphants at 24 hpf (n = 21/21 and n = 13/13, respectively) (Fig. 4G, H and 5C, D). We also analyzed the expression of shh, which is normally detected in the hypothalamus, ZLI, and floor plate. The ZLI, which is located in the intrathalamic boundary, may locally regulate the development of the ventral and dorsal thalamus through Hh signaling [58,59]. The expression of shh in the ZLI was reduced in fgf16 morphants (n = 14/14) (Fig. 4I, J). On the other hand, the expression of shh in the hypothalamus and floor plate was unaffected in fgf16 morphants (n = 14/14) (Fig. 4I, J). Thus, these results indicated that fgf16 was required for the formation of the ZLI and development of the ventral thalamus, but not for the establishment of the dorsal thalamus.

fgf16 was not required for patterning in the midbrain

The MHB is the most characterized local organizing center and is crucial for induction and patterning in the midbrain [60–62]. Fgf8 was previously shown to be required for MHB development and is involved in cell proliferation in the midbrains of chicks [63]. fgf16 morphants showed morphological abnormalities in the MHB constriction and midbrain. Therefore, to investigate whether fgf16 was involved in MHB development, we examined the expression of fgf8 in fgf16 morphants at 24 hpf. The expression of fgf8 was detected in the MHB of fgf16 morphants (n = 27/27) (Fig. 5A, B), which indicates that the MHB is normally formed in fgf16 morphants.

We then investigated whether fgf16 was involved in specification of the midbrain. Otx2 is an important player in the regulation of midbrain patterning [64,65]. The expression of otx2 was unaffected in the midbrains of fgf16 morphants at 24 hpf (n = 13/13) (Fig. 5C, D). Furthermore, we investigated whether fgf16 played a role in the specification of tectal and tegmental fates. The expression of pax7a and nkh6.2 was also unaffected in the tectum and tegmentum in fgf16 morphants at 24 hpf (n = 13/14 and n = 17/17), respectively (Fig. 5E–H). These results demonstrated
that tectal and tegmental characteristics were not affected by *fgf16* MO, and also suggested that the morphological abnormalities observed in the midbrains of *fgf16* morphants may have been due to decreases in cell proliferation.

**fgf16** was required for GABAergic neuron and oligodendrocyte development, but not for that of glutamatergic neuron

In addition to patterning in the brain, Fgfs are involved in the development of neuronal subpopulations [8,66,67]. To determine whether an injection of *fgf16* MO affected neuronal differentiation in the forebrain, the expression of the basic helix-loop helix (bHLH) proneural gene, *ngn1*, was analyzed in *fgf16* morphants at 24 hpf. The expression of *ngn1* was unaffected in the dorsal telencephalon of *fgf16* morphants, whereas it was reduced in the diencephalon (*n* = 11/11) (Fig. 6A, B). We then examined whether the injection of *fgf16* MO affected the expression of *isl1*, a neuronal marker gene, in the forebrain. In the forebrain, *isl1* is expressed by ventral neurons in the telencephalon and diencephalon, and by neurons in the epiphysis at 24 hpf. The expression of *isl1* was reduced in the ventral telencephalon, anterior ventral thalamus, and epiphysis of *fgf16* morphants (*n* = 15/20) (Fig. 6C, D). These results indicated that neuronal differentiation in the ventral region in both the telencephalon and diencephalon was suppressed in *fgf16* morphants.

GABAergic interneurons were previously shown to be generated in the subpallial telencephalon and ventral thalamus of the forebrain [66–71]. *gad1* encoding glutamic acid decarboxylase was found to be expressed specifically in GABAergic interneurons [42]. To examine whether the knockdown of *fgf16* had any effects on GABAergic interneuron differentiation in the forebrain, *gad1* expression was analyzed in *fgf16* morphants at 28 hpf. *gad1* was expressed in the subpallial telencephalon and nucleus of the tract of the postoptic commissure (nTPOC) in the forebrain [42]. In *fgf16* morphants, the expression of *gad1* was severely reduced in both the ventral telencephalon and the nTPOC (*n* = 27/28) (Fig. 6E, F). We also investigated whether GABAergic neurons fully differentiated in *fgf16* morphants. GABA-immunoreactive cell bodies were not detected in the forebrains of *fgf16* morphants at 3 dpf (*n* = 20/20) (Fig. 7A, B). Oligodendrocytes in the telencephalon also originated from the subpallial domain [70]. To investigate the involvement of *fgf16* in oligodendrocyte specification, we examined the expression of *olig2*, a marker of the oligodendrocyte precursor, in *fgf16* morphants at 28 hpf. In addition to the subpallial telencephalon, *olig2* was also shown to be expressed in the ventral thalamus and dorsal thalamus [44]. In *fgf16* morphants, the expression of *olig2* was significantly reduced in the subpallial telencephalon, ventral thalamus, and dorsal thalamus (*n* = 14/20) (Fig. 6G, H). Furthermore, we determined whether *fgf16* was involved in the formation of myelinating oligodendrocytes. PLP (proteolipid protein)/DM20 is a marker of oligodendrocyte differentiation and is expressed in newly formed oligodendrocyte progenitor cells, well before myelination [72–74]. The expression of *plp* was not detected in the forebrains of *fgf16* morphants at 4.5 dpf (*n* = 12/12) (Fig. 7C, D). In addition, the expression of *plp* in the hindbrain disappeared in *fgf16* morphants at 4.5 dpf (*n* = 10/12) (Fig. 7C, D). The immunoreactivity of CC1/APC, which is normally detected in mature oligodendrocyte cell bodies, was also lost in the hindbrains of *fgf16* morphants at 4.5 dpf (*n* = 11/11) (Fig. S2A, B). These results demonstrated that the specification and differentiation of GABAergic interneurons and oligodendrocytes in the forebrain was suppressed in *fgf16* morphants. We investigated whether the knockdown of *fgf16* had any effects on the differentiation of glutamatergic neurons generated in the pallial telencephalon [75]. The expression of *slc17a6a*/*vesicular glutamate transporter (vglut) 2.2*, the postmitotic marker of glutamatergic neurons, was analyzed in *fgf16* morphants at 28 hpf. In *fgf16* morphants, the expression of *slc17a6a* was unaffected in both the pallial telencephalon and

---

**Figure 4. Telencephalic and diencephalic gene expression in the *fgf16* morphants.** The expression of *emx1* (A, B), *tbr1* (C, D), *dlx2* (E, F), *pax6a* (G, H), and *shh* (I, J) in wild-type embryos (A, C, E, G, I) and *fgf16* morphants (B, D, F, H, J) at 24 hpf. Arrows in panels A and C indicate the subpallial telencephalon, which was negative for *emx1* or *tbr1*. Dots indicate the boundary between the telencephalon and ventral diencephalon. Lateral views with anterior to the left and dorsal to the top.

doi:10.1371/journal.pone.0110836.g004
dierephalon (n = 14/14) (Fig. 6I, J). This result demonstrated that glutamatergic neurons in both the pallial telencephalon and dierephalon were specified in fgf16 morphants. Thus, fgf16 was required for the specification and differentiation of GABAergic neurons and oligodendrocytes, but not for that of glutamatergic neuron in the forebrain.

Hh signaling was required for fgf16 expression in the brain

Hh signaling in the ventral forebrain functions in dorsoventral (D/V) forebrain patterning and promotes the GABAergic neuronal/oligodendrocyte lineage restriction of forebrain stem cells [44,76,77]. The inhibition of fgf16 led to abnormalities in the regionalization and generation of specific cell types such as GABAergic interneurons and oligodendrocytes in the forebrain. Hh signaling is critical for regulating the expression of fgf3, fgf8, and fgf19 in the forebrain and that of fgf19 and fgf22 in the midbrain [8,9]. Therefore, we examined whether the expression of Fgf16 was responsive to Hh signaling. Since the alkaloid cyclopamine completely blocked Hh signaling at the level of Smoothened, which transduces Hh signals, in zebrafish [8,78], we examined the expression of Fgf16 in embryos treated with cyclopamine. In embryos treated with cyclopamine, fgf16 expression was lost in the forebrain at 16 and 25 hpf (n = 16/16 and n = 10/10, respectively) (Fig. 8A–D). Furthermore, fgf16 expression in the midbrain was lost in embryos treated with cyclopamine (n = 10/10) (Fig. 8C, D). All control embryos showed normal expression patterns for these genes (Fig. 8A, C).
morphant, but not in A–D) The expression of genitor cells.
neurons, GABAergic interneurons, and oligodendrocyte pro-
wild-type embryos (A, C) and
during forebrain development, we examined its expression in fgf3/8 double morphant embryos at 24 hpf. The expression of fgf16 was unaffected in the forebrains of fgf3/8 double morphant embryos (n = 22/24) (Fig. 9A, B). In contrast to the forebrain, an injection of both Fgf3 MO and Fgf8 MO led to a reduction in the expression of fgf16 in the midbrain at 24 hpf (n = 22/24) (Fig. 9A, B). This result indicated that the combinatorial function of fgf3 and fgf8 was involved in regulating fgf16 expression in the midbrain, but not in the forebrain. In addition to fgf3 and fgf8, fgf19 is required for the regional patterning and specification of GABAergic interneurons and oligodendrocytes in the forebrain [8]. Furthermore, fgf19 regulates the growth of the forebrain and midbrain [8]. The phenotype of fgf16 morphants was essentially similar to that of fgf19 morphants. Therefore, we also examined whether fgf16 expression was affected in fgf19 morphants. fgf16 expression in both the forebrain and midbrain was reduced in fgf19 morphants at 24 hpf (n = 12/14) (Fig. 9A, C). Thus, fgf16 expression in the forebrain was regulated by the function of fgf19, but not by the combinatorial function of Fgf3 and Fgf8. On the other hand, fgf16 expression in the midbrain was dependent on fgf3, fgf8, and fgf19.

Discussion

Roles of fgf16 in cell proliferation during brain development

Fgf signaling regulates the proliferation and differentiation of specific neuronal cell types in the forebrain and midbrain [8,55,56,66]. Fgf8 is required for MHB development, and the MHB is crucial for proliferation and patterning in the midbrain [60–63]. However, fgf8 has not been implicated in growth of the forebrain [7]. On the other hand, fgf16 knockdown significantly inhibited cell proliferation and led to a reduction in the size and morphological abnormalities in the forebrain and midbrain. fgf16 morphants showed normal expression patterns of fgf19 in the MHB and had normal MHB-specific characteristics. This result indicated that a decrease in cell proliferation in the midbrains of fgf16 morphants was not due to a defect in the MHB. Thus, fgf16 functions are required to promote cell proliferation in the forebrain and midbrain.

Roles of fgf16 in regional patterning during brain development

The expression of fgf16 was first detected in the most anterior part of the ventral telencephalon at 14 hpf. fgf16 morphants exhibited the expanded expression of markers for the pallial telencephalon, enx1 and tbr1, and decreased expression of markers for the subpallial telencephalon, dlx2, at 24 hpf. These results suggested the loss of subpallial fate in the telencephalon of fgf16 morphants. Reduced cell proliferation in the telencephalon was observed in fgf16 morphants. Therefore, subpallial cells may be formed in smaller numbers due to reduced cell proliferation caused by the inhibition of fgf16. However, the expanded expression of ngn1 and slc17a6a was not detected in the ventral telencephalon of fgf16 morphants, which suggested that ventral cells in the telencephalon of fgf16 morphants were not formed in...
smaller numbers. Furthermore, fgf16 knockdown did not appear to transform cell fate specification from subpallial to pallial cells, and did not induce differentiation into dorsal neuronal cell types in the subpallial telencephalon. The ectopic expression of otx2 was detected in the ventral telencephalon of fgf16 morphants. Thus, Fgf16 is involved in patterning of the ventral forebrain, whereas the ventral telencephalon does not develop into the pallium following the inhibition of fgf16.

In the diencephalon, the expression of dlx2 was decreased in the ventral thalamus by the inhibition of fgf16 at 24 hpf, whereas that of shh was unaffected in the ventral region. Furthermore, the expression of pax6a was normally detected in the diencephalon of fgf16 morphants at 24 hpf. These results demonstrated that the ventral thalamus was initially induced in fgf16 morphants. Therefore, fgf16 is necessary for maintaining of the characteristics of the ventral thalamus. In contrast, tectum- and tegmentum-specific characteristics were unaffected in the midbrains of the fgf16 morphants. This result indicated that fgf16 may be involved in regulating cell proliferation, but not dorsoventral patterning during midbrain development. In contrast, fgf16 may be involved in both the establishment of the subpallial telencephalon and ventral thalamus as well as the regulation of cell growth during forebrain development.
Roles of fgf16 in specification of GABAergic interneurons and oligodendrocytes in the forebrain

Ngn1 is known to be sufficient for conferring neuronal identity on uncommitted precursors and plays an important role in neuronal differentiation [79–81]. Although Fgf signaling is involved in neuronal differentiation, the expression of ngn1 was unaffected in the dorsal telencephalon of fgf16 morphants. Furthermore, slc17a6a expression was also detected normally in the dorsal telencephalon of fgf16 morphants. On the other hand, the expression of is11 was reduced in the ventral telencephalon, anterior ventral thalamus, and epiphysis, which suggested that fgf16 may be involved in neuronal differentiation in the ventral region, but not the dorsal region in the forebrain. However, slc17a6a expression was detected normally in the ventral thalamus of fgf16 morphants. These results indicated that fgf16 was not required for the specification of glutamatergic neurons in the forebrain.

The expression of dlx2 was reduced in the forebrains of fgf16 morphants. Dlx2 was shown to be involved in the specification of GABAergic interneurons and oligodendrocytes in the telencephalon [82]. Dlx2 is known to induce the GABAergic marker, GAD1, when ectopically expressed in cortical explants [83]. olig2, expressed in oligodendrocyte precursors, is necessary and sufficient for the generation of oligodendrocytes throughout the neuraxis [44,84,85]. fgf16 knockdown resulted in a severe reduction of the expression of gad1 and olig2 in the ventral telencephalon and diencephalon. GABA-immunoreactive cells were also lost in the forebrains of fgf16 morphants, which indicated that GABAergic neurons did not fully differentiate in fgf16 morphants. pIpl expression and CC1 immunoreactivity also disappeared in fgf16 morphants, which suggested that the oligodendrocytes did not terminally differentiate into myelinating cells in fgf16 morphants. These results demonstrated that fgf16 was involved in the specification of GABAergic interneurons and oligodendrocytes in the ventral telencephalon and diencephalon. On the other hand, the knockdown of fgf16 did not strongly stimulate apoptosis in the forebrain. This result suggested that the survival of GABAergic interneurons and oligodendrocytes was unaffected by fgf16. Accordingly, Fgf16 appears to be crucial for the differentiation of GABAergic interneurons and oligodendrocytes, but not for that of glutamatergic neurons in the forebrain.

fgf16 was regulated by Hh and Fgf19 signaling in forebrain development

Shh plays a mitogenic role in the brain and the ectopic expression of Hh target genes causes human cancers such as Basal Cell Carcinoma or medulloblastoma, a granule cell tumor [58,86,87]. Cell proliferation in the forebrain and midbrain was decreased in the fgf16 morphants as well as Shh mutant mice. Furthermore, the expression of fgf16 in the forebrain and midbrain was markedly reduced by the inhibition of Hh signaling at 16 and 25 hpf. These results indicated that Fgf16 may function downstream of Hh activity in cell proliferation in the forebrain and midbrain. On the other hand, Fgf8 participates in the growth of the midbrain, whereas Fgf3 and Fgf8 are not required for growth of the forebrain [7,18,63]. Consistent with these findings, the inhibition of both fgf3 and fgf8 led to a reduction in the expression of fgf16 in the midbrain, whereas it was unaffected in the forebrains of fgf3/8 double morphant embryos. Thus, fgf3 and fgf8 expressed in the MHB may regulate cell proliferation in the midbrain by activating the expression of fgf16 in the midbrain.

In addition to cell proliferation, Hh signaling is required for patterning in the telencephalon and the generation of GABAergic neuronal/oligodendrocyte progenitors from ventral forebrain stem cells via the activation of olig2 [3,44,76,77]. fgf16 morphants as well as smu/smo mutants exhibited the suppressed specification of GABAergic interneurons and oligodendrocytes in the forebrain. Hh signaling specifies GABAergic interneurons and oligodendrocytes via fgf3, fgf8, and fgf19 in the ventral forebrain, and this ensures the expression of pan-ventral transcription factors, such as dlx2 and olig2, whereas Fgf19 has distinct functions independent from those of Fgf3 and Fgf8 [8]. The inhibition of fgf19 led to a reduction in the expression of fgf16 in the forebrain, whereas the expression of fgf16 was unaffected in fgf3/8 double morphant embryos. This result indicates that fgf16 expression in the forebrain is regulated by Fgf19, but not by Fgf3/Fgf8. Thus, the effects of Hh activity on the differentiation of GABAergic interneurons and oligodendrocytes may be mediated through Fgf19-Fgf16 pathways in the forebrain.

In conclusion, the present results indicated that fgf16 expressed in the developing brain plays crucial roles in brain development. fgf16 is involved in cell proliferation in the forebrain and midbrain. fgf16 is also involved in the development of the ventral region and specification and differentiation of GABAergic interneurons and oligodendrocytes in the forebrain. On the other hand, fgf16 was not required for the specification of tectal and tegmental fates. Furthermore, the expression of fgf16 was dependent on Hh and fgf19. The present results suggest that crosstalk between Fgf16 signaling and Fgf19 and Hh signaling may be crucial for cell proliferation, regionalization, and cell type specification during forebrain development.

Supporting Information

Figure S1 Apoptosis in the brain of fgf16 morphants. At 24 hpf, apoptotic cells in the brain of the wild-type (A) and fgf16 MO1-injected (B) embryos were marked via TUNEL. Lateral views with anterior to the left and dorsal to the top.

Figure S2 Oligodendrocyte differentiation in the hindbrain of fgf16 morphants. (A, B) Dorsal views of wild-type embryos (A) and
fgf16 morphants (B), labeled to show CC1/APC immunoreactivity at 4.5 dpf.

(TIF)

Author Contributions

Conceived and designed the experiments: A Miyake YN MK NI. Performed the experiments: A Miyake TC EK A Murakami. Analyzed the data: A Miyake TC EK YN MK NI. Contributed to the writing of the manuscript: A Miyake NI.

References

1. Wilson SW, Houart C (2004) Early Steps in the Development of the Forebrain. Dev Cell 6: 167–181.
2. Wilson SW, Rubenstein JLR (2000) Induction and Dorsal-ventral Patterning of the Telencephalon. Neuron 28: 641–653.
3. Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, et al. (1996) Cytoadhesion and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature 383: 407–413.
4. Bilic B, Kajstura J, Bresciani TM, Sirault G (2001) A hedgehog-inhibitive form of patched provides evidence for direct long-range morphogen activity of sonic hedgehog in the neural tube. Mol Cell 7: 1279–1291.
5. Varga ZM, Amores A, Lewis KE, Yan YL, Postlethwait JH, et al. (2001) Fgfr gene families. Trends Genet 17: 563–569.
6. Lu SY, Sontag DP, Detillieux KA, Cattini PA (2008) FGF-16 is released from Timp2 and required for cardiomyocyte proliferation in the mouse embryonic heart. Dev Biol 318: 193–207.
7. Park H-C, Mehta A, Richardson JS, Appel B (2002) FK506BP, a CNS-specific DISC1 binding protein, positively regulates oligodendrocyte differentiation. Glia 42: 335–346.
8. Ishii H, Kato S, Kinoshita H, Takeda H, Takeda K, et al. (2003) CKIIalpha promotes adhesion molecules expression and neurite outgrowth of embryonic and fetal dorsal root ganglionic neurons. J Neurosci Res 71: 318–327.
9. Matsumoto E, Sasaki S, Kinoshita H, Kato T, Obata H, et al. (2013) Angiostatin II-induced cardiac hypertrophy and fibrosis are promoted in mice lacking Fgf16. Genes Cells 18: 544–553.
10. Nomura R, Kamei E, Hotta Y, Konishi M, Miyake A, et al. (2006) Fgf16 is essential for pectoral fin bud formation in zebrafish. Biochem Biophys Res Comm 340: 346–349.
11. Koshida S, Shinya M, Ohi T, Kuroiwa A, Takeda K (2004) The Fgf16 mutant exhibits pronounced craniofacial defects with severe cardiovascular anomalies. Dev Dyn 229: 609–617.
12. Shima M, Yamashita M, Miyake A, Konishi M, Koyama Y, et al. (2007) Cloning of zebrafish fgf16, an RGD-containing growth factor, and expression analysis in zebrafish embryos. Dev Dyn 236: 2980–2991.
13. Shima M, Yamashita M, Miyake A, Konishi M, Koyama Y, et al. (2005) Fgf16 promotes the formation of the otic placode in zebrafish. Development 132: 1117–1123.
14. Nakamura T, Yamashita M, Itoh N, Konishi M, Koyama Y, et al. (2003) Fgf16 is required for cardiac myocyte proliferation and angiogenesis in the embryonic heart. Development 130: 3825–3837.
15. Kajstura J, Bresciani TM, Sirault G, Hammarlund U, Saldias J, et al. (2001) FGF16 is required for cardiac myocyte proliferation in the mouse embryonic heart. Dev Dyn 227: 1969–1979.
16. Lu SY, Dong Q, Cattini PA (2000) Cloning of a novel member, FGF-16, on the fibroblast growth factor (FGF) gene family. Biochem Biophys Res Commun 273: 448–452.
17. Hatch EP, Umeson LD, Mansour SL (2009) Fgf16(IRESCre) mice: a tool to inactivate genes expressed in inner ear cristae and spiral prominence epithelium. Dev Dyn 238: 356–366.

20. Maves L, Jackman W, Kimmel CB (2002) FGF3 and FGF8 mediate a shared dorsoventral and mediolateral signaling activity. Development 129: 1211–1222.
54. Incardona JP, Gaffield W, Kapur RP, Roelink H (1998) The teratogenic
Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction.
Development 125: 3533–3562.
55. Xu J, Liu Z, Ornitz DM (2000) Temporal and spatial gradients of Fgf8 and
Fgf17 regulate proliferation and differentiation of midline cerebellar structures.
Development 127: 1833–1843.
56. Trokovic R, Trokovic N, Hermenseni S, Pivovska U, Vogt Weisenhorn DM, et
al. (2003) FGFRI is independently required in both developing mid- and
hindbrain for sustained response to isthmic signals. EMBO J 22: 1111–1123.
57. Hendzel MJ, Wei Y, Mancini MA, Van Hooser A, Ranalli T, et al. (1997)
Mitosis-specific phosphorylation of histone H3 initiates primarily within
pericentromeric heterochromatin during G2 and spreads in an ordered fashion
coincident with the mitotic chromosome condensation. Chromosoma 106: 348–
360.
58. Ishibashi M, McMahon AP (2002) Asonic hedgehog-dependent signaling relay
regulates growth of diencephalic and mesencephalic primordial in the early
mouse embryo. Development 129: 4897–4918.
59. Hashimoto-Tori K, Motoyama J, Hui CC, Kuroiwa A, Nakafuku M, et al.
(2003) Differential activities of Sonic hedgehog mediated by Gli transcription
factors define distinct neuronal subtypes in the dorsal thalamus. Mech Dev 120:
1097–1111.
60. Martinez S (2001) The isthmic organizer and brain regionalization. Int J Dev
Biol 45: 367–371.
61. Rhinn M, Brand M (2001) The midbrain-hindbrain boundary organizer. Curr
Opin Neurobiol 11: 34–42.
62. Wurst W, Bally-Cuif L (2001) Neural plate patterning: upstream and
downstream of the isthmic organizer. Curr Opin Neurobiol 11: 34–42.
63. Sato T, Araki I, Nakamura H (2001) Inductive signal and tissue responsiveness
downstream of the isthmic organizer. Nat Rev Neurosci 2: 99–108.
64. Katahira T, Sato T, Sugiyama S, Okafuji T, Araki I, et al. (1998) FGF and
Shh signals control dopaminergic and serotonergic cell fate in the anterior
neural plate. Cell 93: 755–766.
65. Ye W, Shimamura K, Rubenstein JL, Hynes MA, Rosenthal A (1998) FGF and
Shh signals control dopaminergic and serotonergic cell fate in the anterior neural
plate. Cell 93: 755–766.
66. Bosco A, Bureau C, Affatati P, Gaspar P, Bally-Cuif L, et al. (2013)
Development of hypothalamic serotoninergic neurons requires Fgf signalling via
the ETS-domain transcription factor En1b. Development 140: 372–384.
67. Corbin JG, Nery S, Fishell G (2001) Tenecephalic cells take a tangential:
nonradial migration in the mammalian forebrain. Nat Neurosci 4: 1177–1182.
68. Jones EG (2001) Dichotomous appearance and unusual origins of GABA neurons
during development of the mammalian thalamus. Thalamus Relat Syst 1: 283–
288.
69. Marin O, Rubenstein JLR (2001) A long, remarkable journey: tangential
migration in the telencephalon. Nat Rev Neurosci 2: 780–790.
70. Ruiz i Altaba A, Sanchez P, Dahalane N (2002) Gli and hedgehog in cancer:
tumours, embryos and stem cells. Nat Rev Cancer 2: 361–372.