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Inductive signal and tissue responsiveness defining the tectum and the cerebellum

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SUMMARY

The mes/metencephalic boundary (isthmus) has an organizing activity for mesencephalon and metencephalon. The candidate signaling molecule is Fgf8 whose mRNA is localized in the region where the cerebellum differentiates. Responding to this signal, the cerebellum differentiates in the metencephalon and the tectum differentiates in the mesencephalon. Based on the assumption that strong Fgf8 signal induces the cerebellum and that the Fgf8b signal is stronger than that of Fgf8a, we carried out experiments to misexpress *Fgf8b* and *Fgf8a* in chick embryos. Fgf8a did not affect the expression pattern of *Otx2, Gbx2* or *Irx2*. En2 expression was upregulated in the mesencephalon and in the diencephalon by Fgf8a. Consequently, *Fgf8a* misexpression resulted in the transformation of the presumptive diencephalon to the fate of the mesencephalon. In contrast, Fgf8b repressed *Otx2* expression, but upregulated *Gbx2* and *Irx2* expression in the mesencephalon. As a result, Fgf8b completely changed the fate of the mesencephalic alar plate to cerebellum. Quantitative analysis showed that Fgf8b signal is 100 times stronger than Fgf8a signal. Co-transfection of *Fgf8b* with *Otx2* indicates that Otx2 is a key molecule in mesencephalic generation. We have shown by RT-PCR that both *Fgf8a* and *Fgf8b* are expressed, *Fgf8b* expression prevailing in the isthmic region. The results all support our working hypothesis that the strong Fgf8 signal induces the neural tissue around the isthmus to differentiate into the cerebellum.

Key words: Fgf8, Cerrebellum, Tectum, Mesencephalon, Metencephalon, Cell signalling, Chick

INTRODUCTION

Molecular mechanisms involved in the regionalisation of the vertebrate central nervous system (CNS) have been a focus of many studies. A combination of transcription factors and inducing signals from the regional boundary may determine the fate of the region (reviewed by Nakamura, 2000). Loss-of-function and gain-of-function studies of *Otx2, En1/2, Pax2/5* suggest that these genes are indispensable transcription factors for mesencephalic development. Transplantation experiments have indicated that the mes/metencephalic boundary (isthmus) has an organizing activity for the mesencephalon and metencephalon (Martinez et al., 1991; Martinez et al., 1995). When the isthmic region was transplanted to the diencephalon, the transplant induced En expression, and surrounding tissue differentiated into the tectum (Martinez et al., 1991). When the isthmus was transplanted in the rhombencephalon, the transplant also induced En but the surrounding tissue differentiated into the cerebellum (Martinez et al., 1995). The tectum and the cerebellum, which differentiate from the mesencephalic and the metencephalic alar plate respectively, are exposed to the isthmic organizing signal, but their mode of differentiation is quite different (Jacobson, 1991; LaVail and Cowan, 1971). Consequently, histological architecture of the tectum and the cerebellum is different.

The molecular nature of the organizing signal and the responsiveness of the tissue to the signal are of great interest. It is accepted that Fgf8 provides the organising activity for the mesencephalon since Fgf8-soaked beads implanted into the diencephalon mimics the isthmic organizer (Crossley et al., 1996; Martinez et al, 1999; Shamim et al., 1999). It was reported that *Fgf8* expression is induced at the interface of Otx2 and Gbx2 expression domains overlapping with the Gbx2 domain (Hidalgo-Sanchez et al., 1999; Katahira et al., 2000; Simeone, 2000), where the cerebellum differentiates. We hypothesised that strong Fgf8 signal induces cerebellum. There are 8 Fgf8 isoforms identified so far (Crossley and Martin, 1995; MacArthur et al., 1995a), and it has been reported that Fgf8b has stronger transforming activity than Fgf8a (MacArthur et al., 1995b). Recently, it was reported that Fgf8b could induce a metencephalic phenotype in the diencephalon or in the mesencephalon (Martinez et al., 1999; Liu et al., 1999). We compared the effect of Fgf8a and Fgf8b by in ovo electroporation, a strong gene transfer system (Funahashi et al., 1999). We first examined Fgf8a and Fgf8b expression at the isthmic region by reverse transcription polymerase chain reaction (RT-PCR), then compared the organizing activity of Fgf8a and

Fgf8b by misexpression using in ovo electroporation (Funahashi et al., 1999). We found that Fgf8b completely transformed the mesencephalic alar plate to differentiate as cerebellum. We then carried out quantitative experiments to ascertain if the type-difference in the effect of Fgf8 could be ascribed to the difference in the strength of the signal. We also paid special attention to the expression of *Otx2*, *Gbx2* and *c-Irx2* as these genes may determine the responsiveness to the organizing signal.

MATERIALS AND METHODS

Probes, cDNAs and expression vectors

Fgf8a, *Fgf8b* cDNAs were cloned by PCR from mRNA of embryonic day (E) 3 chick embryos. Probes for *En1*, *Pax6*, *Pax2*, *Pax5* and *Gbx2* were isolated as described previously (Araki and Nakamura, 1999; Itasaki and Nakamura, 1996; Katahira et al., 2000; Okafuji et al., 1999) *Otx2* and *Irx2* probes are kind gifts from Drs Kitamura and Ogura, respectively. *Fgf8a*, *Fgf8b* and *Otx2* cDNAs were inserted in the expression vector, pMiwSV, which has a chick β-actin promoter and RSV enhancer (Suemori et al., 1990; Wakamatsu et al., 1997)

In ovo electroporation

In ovo electroporation was carried out as described previously (Funahashi et al., 1999; Nakamura et al., 2000). Briefly, fertilized chicken embryos were incubated in humid condition at 38ºC for 36 hours to reach stage 10 (Hamburger and Hamilton, 1951). DNA solution was injected into the lumen of the neural tube. The electrodes (Unique Medical Imada, Natori, Japan) were placed on the vitelline membrane 4 mm apart, then a rectangular pulse of 25 V, 50 mseconds was given 4 times by electroporation (CUY21, Tokiwa Science, Fukuoka, Japan). To monitor the ectopic expression, a GFP expression vector was added to the DNA solution (0.35 μg/μl). Only the anode side of the neural tube is transfected, as DNA is negatively charged. The other side was used as a control. Since transfection on younger embryos (stage 7-9) gave similar results to embryos at stage 10, the latter embryos were used throughout because of better survival rates.

RT-PCR

Poly(A)$^+$ RNA was isolated from the isthmic region of stage 9-10 embryos with QuickPrep® mRNA Purification kit (Amersham Pharmacia Biotech). Reverse transcription was performed with SuperScript™II RT (Gibco BRL®), and cDNA was amplified by PCR with primers that can distinguish the Fgf8 subtypes. The primer set used in the present study was: 5′-TTCATGCACTTGTTCGTCC-3′ and 5′-TCTCGACGATGAGCTTGG-3′. For the quantitative analysis, PCR product was harvested every 4 cycles, and the amount of amplified DNA was analysed by NIH Image.

In situ hybridization

Whole-mount in situ hybridization was carried out according to the method of Bally-Cuif et al. (Bally-Cuif et al., 1995). For double-colored in situ hybridization, one probe was labeled with fluorescein isothiocyanate (FITC), and the other was labeled with digoxigenin (DIG) according to the manufacturer's protocol (Promega). Alkaline phosphatase (ALP)-conjugated anti-FITC and anti-DIG antibodies (Roche Diagnostics KK) were colored with Fast Red/Naphthol AS/MX (red), and nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (purple), respectively. In some cases, the red color was removed with ethanol to show the effect clearly.

Immunohistochemistry

Monoclonal antibodies used were: anti-En2 antibody, 4D9 (American Type Culture Collection), anti-neurofilament antibody, 3A10, anti-Islet1 antibody, 40.3A4 (Developmental Studies Hybridoma bank), and anti-calbindin antibody (Sigma). As a second antibody, horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Jackson), or Cy3-conjugated anti-mouse IgG (Jackson) was used. DAB (3,3′-diaminobenzidine) was adopted as the chromogen for HRP. Biotinylated anti-mouse IgG was also used as the second antibody, and immunoreactivity was detected using the ABC-Elite system. Immunostaining was carried out after in situ hybridization.

Histology

Embryos were fixed with 4% paraformaldehyde, and embedded in Historesin (Leica). Serial 5 μm sections were stained with Hematoxylin and Eosin.

RESULTS

Fgf8a and Fgf8b are expressed in isthmus

In mice, there are at least 6 exons in the *Fgf8* gene, which are alternatively spliced to give potentially eight different Fgf8 isoforms (Crossley and Martin, 1995; MacArthur et al., 1995a). To identify the isoforms of *Fgf8* expressed in the isthmus of chick embryos, we designed primer sets (Fig. 1A) that could distinguish Fgf8 isoforms by PCR, and carried out RT-PCR on mRNA isolated from the isthmic region of E1.5 (embryonic day 1.5) chick embryos. Both *Fgf8a* and *Fgf8b* were detected (Fig. 1B). Since PCR was performed using the same primer pairs, differences in the quantity of PCR product before saturation may reflect quantitative difference in the amount of mRNA expressed in the isthmus. By analysis of Fig. 1B by NIH Image, it was shown that both DNA was amplified logarithmically and with equal efficiency until 40 cycles, and saturated around 44 cycles. Quantitative analysis shows that the level of *Fgf8b* expression was much higher than that of *Fgf8a* (Fig. 1B) Other isoforms were not detected.

Misexpression of Fgf8a transformed the presumptive diencephalon to the fate of mesencephalon

We misexpressed *Fgf8a* and *Fgf8b* on the right side of the brain vesicles by in ovo electroporation at stage 10. The left side served as the control. At E6.5-7.5 (stage 30-32) tectal swelling on the experimental side extended anteriorly and became larger than that on the control side (8/8, Fig. 2A). Histologically, the alar plate of the prospective diencephalon appeared to have differentiated into the tectum as a result of *Fgf8a* misexpression (Fig. 2B). Anterior extension of the tectal swelling was already discernible at E3.5 (stage 21, Fig. 2C). A trunk of the oculomotor nerve arose from the basal plate of the mesencephalon on the control side (Fig. 2D). On the transfected side, additional nerve trunks running similarly to the oculomotor nerve were apparent (3/3, Fig. 2E), suggesting that the basal plate of the diencephalon was transformed into that of mesencephalon. Consistent with this idea, expression of Islet1, which is expressed in the oculomotor nucleus on the control side, extended more anteriorly in the prospective diencephalic territory (Fig. 2F). The trochlear nucleus was not affected (Fig. 2F), indicating that Fgf8a did not affect isthmic development.

Fgf8b transformed the alar plate of the mesencephalon to the cerebellum

Misexpression of *Fgf8b* produced drastic effects on the mesencephalon. The tectal swelling of the transfected side of

E13.5-E15.5 (stage 39-42) embryos was smaller than that of the control side, and had fissures that are typical for the cerebellum (12/12, Fig. 3A-C). Histologically, a well differentiated external granular layer was present (Fig. 3D-F), and beneath it was a layer with large cell bodies, which resembled the Purkinje cell layer of the normal cerebellum (Fig. 3E,F). Immunoreactivity to anti-calbindin antibody, a marker for Purkinje cells in the cerebellum, confirmed that these large cells were Purkinje cells (Fig. 3G,H). Thus, we concluded that Fgf8b transformed the alar plate of the prospective mesencephalon to the cerebellum instead of differentiating into the tectum. In E3.5 embryos (stage 21), in which morphological alteration was already obvious (5/5, Fig. 3I), the oculomotor nerve trunk was missing in the ventral mesencephalon on the transfected side (3/3, Fig. 3J,K). The oculomotor, or trochlear, nucleus did not exist at its proper site, whereas one nucleus was identified at the diencephalic level on the transfected side (Fig. 3L). These data suggest that both the alar plate and the basal plate of the prospective mesencephalon were transformed into metencephalon by *Fgf8b* misexpression.

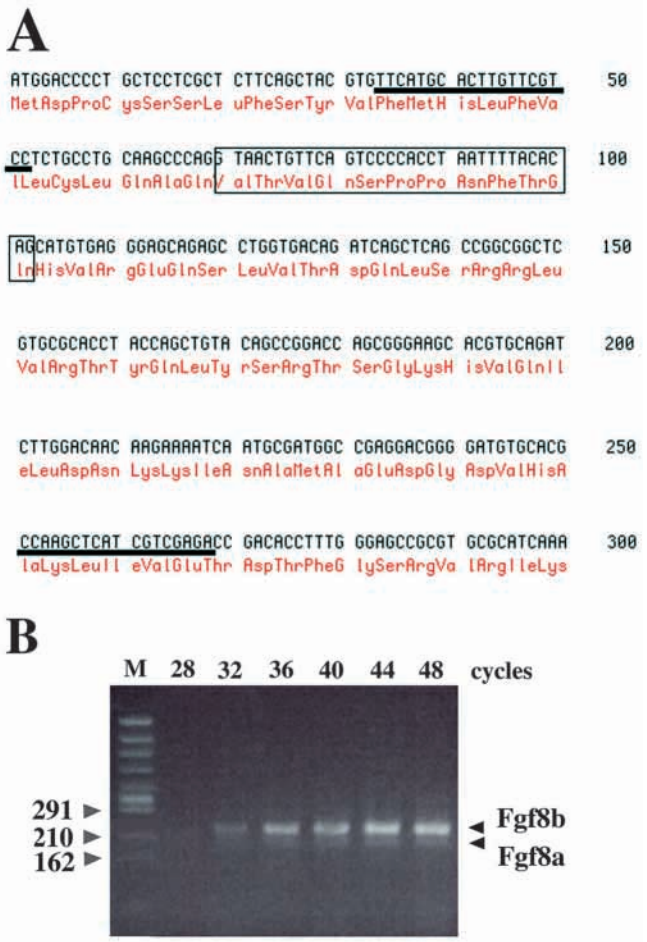

Fig. 1. Analysis of *Fgf8a* and *Fgf8b* in the isthmus by RT-PCR. (A) Part of the *Fgf8b* cDNA and amino acid sequence showing the difference between *Fgf8a* and *Fgf8b*. *Fgf8a* lacks the boxed region (Crossley and Martin, 1995; MacArthur et al., 1995a). The primer pair for RT-PCR are underlined, expected length of Fgf8a and Fgf8b being 202 and 235, respectively. (B) Quantitative RT-PCR. The number above each lane indicates the number of the cycles of PCR. Lane M is the DNA size marker of a ϕx174/*Hin*cII digest. Analysis by NIH Image based on this figure indicated that amplification was logarithmic until cycle 40. RT-PCR analysis shows that *Fgf8a* and *Fgf8b* are localized in the isthmic region, *Fgf8b* being predominant. Other *Fgf8* isoforms were not detected.

Effects of Fgf8a and Fgf8b misexpression on mes/metencephalon-related molecules

Next, we examined the effects of *Fgf8a* and *Fgf8b* misexpression on the mes/metencephalon-related molecules. First, we examined the effects on the expression of *Otx2* and *Gbx2*. It has been shown that repressive interaction between Otx2 and Gbx2 determines the location of the mes/metencephalic boundary (Broccoli et al., 1999; Millet et al., 1999; Katahira et al., 2000). Misexpression of *Otx2* in the metencephalon changed its fate to mesencephalon (Broccoli et al., 1999; Katahira et al., 2000), which indicates that *Otx2* plays a crucial role in the development of the tectum. Fgf8a did not affect *Otx2* (4/4) or *Gbx2* (9/9) expression at 24 hours after electroporation (stage 17, Fig. 4A-D). In contrast, Fgf8b repressed *Otx2* expression (5/5, Fig. 5A-C), and induced *Gbx2* expression in the mesencephalon and in the caudal

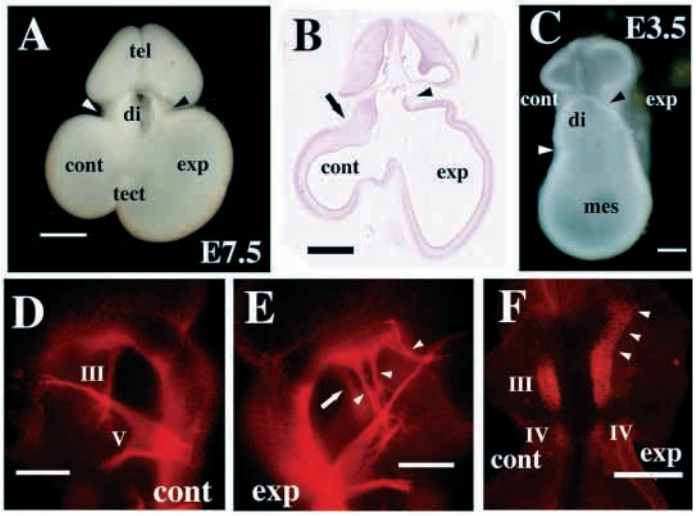

Fig. 2. Effects of Fgf8a misexpression. (A) Dorsal view of the E7.5 chick brain. Misexpression of Fgf8a enlarged the tectum (compare control side and the experimental side). In A-C the anterior limit of the tectum is indicated by white arrowheads and a black arrow (in B) on the control side, and by black arrowheads on the experimental side. (B) Horizontal section showing that the tectum has enlarged into the diencephalic territory. (C) Tectal swelling extended towards anterior at E3.5. (D,E) Whole-mount immunohistochemistry with anti-neurofilament antibody of an E3.5 embryo. On the control side, oculomotor (III) and trigeminal (V) nerves are present. On the experimental side (E), there are 3 nerve trunks that run similarly to the oculomotor nerve (arrowheads). The arrow indicates the oculomotor nerve on the control side. (F) Immunohistochemistry with anti-Islet1 antibody of an E3.5 embryo to show the motor nuclei. On the control side the oculomotor nucleus (III) at the mesencephalon and the trochlear nucleus (IV) at the isthmus are clearly visible. On the experimental side the oculomotor nucleus extends anteriorly into the diencephalic territory (arrowheads). cont: control side; exp: experimental side, tel: telencephalon, di: diencephalon, tect: tectum, mes: mesencephalon. Scale bars, 4 mm (A, B), 800 μm (C), 400 μm (D, E) and 200 μm (F).

diencephalon at 24 hours after electroporation (5/5, Fig. 5D-F).

We then examined the expression of *c-Irx2*, one of chick homologues of *Drosophila Iroquois* expressed in the metencephalon (Goriely et al., 1999). *c-Irx2* expression was not affected by Fgf8a (3/3, Fig. 4E,F), but was induced by Fgf8b in the mesencephalon and in the caudal diencephalon (6/6, Fig. 5G-I). *Pax2* and *Pax5* have been shown to play essential roles in isthmus formation (Krauss et al., 1992; Okafuji et al., 1999; Pfeffer et al., 1998; Funahashi et al., 1999). Misexpression of Fgf8a did not affect *Pax2* (3/3, Fig. 4G,H) or *Pax5* (5/5, Fig. 4I,J) expression. In contrast, *Fgf8b* downregulated the expression of *Pax2* in the isthmus, but induced its expression in the mesencephalon and caudal diencephalon (3/3, Fig. 5J-L).

En1 and En2 are required for regionalisation of the mesencephalon and the metencephalon, and En2 plays an important role in rostrocaudal polarity formation of the tectum (Hanks et al., 1995; Itasaki and Nakamura, 1992; Itasaki and Nakamura, 1996; Wurst et al., 1994). Both Fgf8a and Fgf8b induced *En2* in the caudal diencephalon, mesencephalon and metencephalon, with Fgf8b being the more effective (Figs 4K,L, 5M,N). *En1* was induced by Fgf8a in the anterior mesencephalon and the caudal diencephalon (4/4, Fig. 4M). The posterior limit of the *Pax6* expression domain was shifted anteriorly by Fgf8a (7/7, Fig. 4N,O).

Weaker *Fgf8b* signal exerts similar effects as *Fgf8a*

It has been reported that Fgf8b has stronger transforming activity than Fgf8a (MacAuthur et al., 1995b). We, therefore, tested if weaker Fgf8b signal would exert similar effects as Fgf8a. The expression level of a transgene resulting from in ovo electroporation was shown to depend on the concentration of the DNA solution injected (Momose et al., 1999). We therefore misexpressed *Fgf8b* at concentrations of 1.0, 0.1, 0.01, and 0.001 μg/μl of *Fgf8b* expression vector (Fig. 6). As expected, morphological changes at 0.01 μg/μl were similar to those caused by *Fgf8a*, that is, the diencephalon changed its fate to tectum, and as a result the tectum enlarged (4/4, Fig. 6E,F). The effects on the expressions of molecular markers were also similar to those of Fgf8a (data not shown). These data may justify our working hypothesis that the difference in organizing activity between Fgf8a and Fgf8b is attributable to the difference in the strength of the signal.

Otx2 confers mesencephalic competence

Competence, i.e., tissue responsiveness to the signaling molecule, may be determined by the combination of the transcription factors. It has been shown that *Otx2* and *En1* are indispensable for the tectum development (Araki and Nakamura, 1999; Wurst et al., 1994). Since *En1* is expressed in the metencephalon and in the mesencephalon, we thought that *Otx2* may be a key molecule for the mesencephalic competence. To verify this idea, co-transfection of *Otx2* and *Fgf8b* was carried out (Fig. 7). Misexpression of *Fgf8b* using a 0.1 μg/μl expression vector resulted in complete substitution of the tectum with the cerebellum (4/4, Fig. 6C,D). However, co-transfection of *Fgf8b*

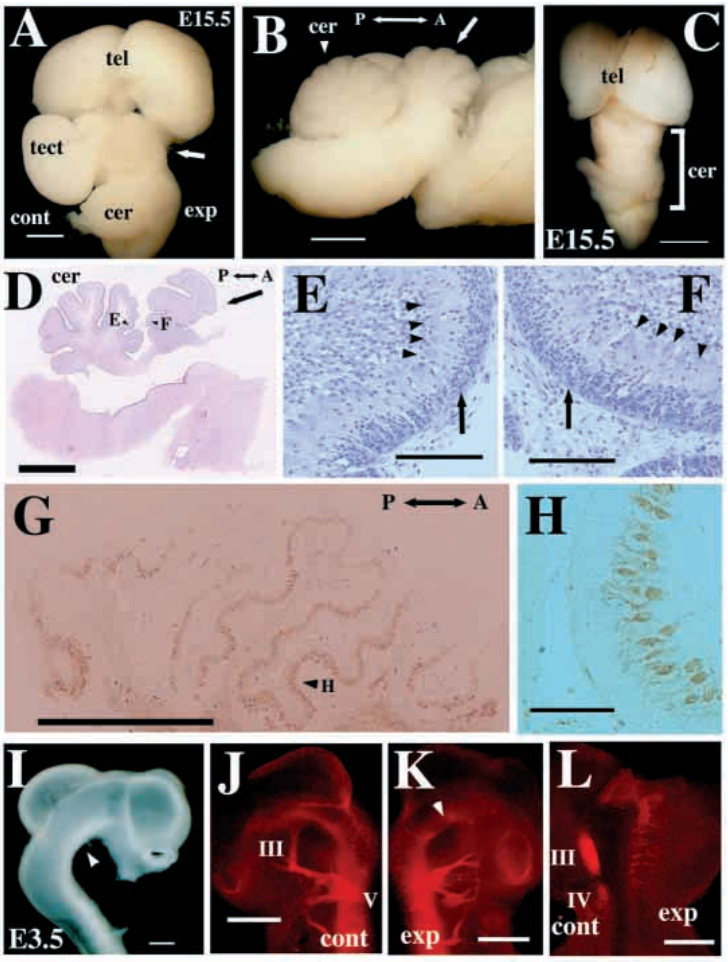

Fig. 3. Effects of Fgf8b misexpression. (A,B) Dorsal and lateral view of an E15.5 brain after misexpression of *Fgf8b*. The tectum has disappeared, and instead cerebellum has differentiated ectopically (arrow in A,B). (C) Misexpression on both sides has completely replaced the tectum with the cerebellum (cer). (D) Parasagittal section of the brain in B to show the cerebellum proper (cer) and the part that differentiated from the mesencephalic alar plate (arrow). (E,F) Higher magnification of the cerebellum proper (cer) and the cerebellum-like structure (indicated as E and F in D). The cerebellar structure in the mesencephalic region has an external granular layer (compare the cells indicated by an arrow in E and F), and the layer that is identical to the Purkinje cell layer (compare arrowheads in E and F). (G,H) Immunocytochemistry with anti-calbindin antibody. Parasagittal cryosection of the brain shown in C was stained with anti-calbindin antibody. H is a higher magnification micrograph of the area indicated in G. Anti-calbindin antibody specifically stains Purkinje cells in the cerebellum, and G and H show that Purukinje cells are differentiated in this structure. (I-L) E3.5 embryos after *Fgf8b* misexpression. (I) Lateral view; there is no tectal swelling (arrowhead). (J,K) Whole-mount immunohistochemistry with anti-neurofilament antibody. (L) Flat-mount specimen after immunohistochemistry with anti-islet-1 antibody. The oculomotor nerve trunk (arrowhead inK) and the nucleus (L) disappeared on the experimental side. cer: cerebellum, cont: control side, di: diencephalon, exp: experimental side, mes: mesencephalon, tect: tectum, tel: telencephalon, III: oculomotor nerve trunk or oculomotor nucleus, IV: trochlear nucleus, V: trigeminal nerve. The anteroposterior direction is indicated by the arrow on B, D and G. Scale bars, 4 mm (A-D,G), 800 μm (I), 400 μm (J,K), 200 μm (L) and 100 μm (E,F,H).

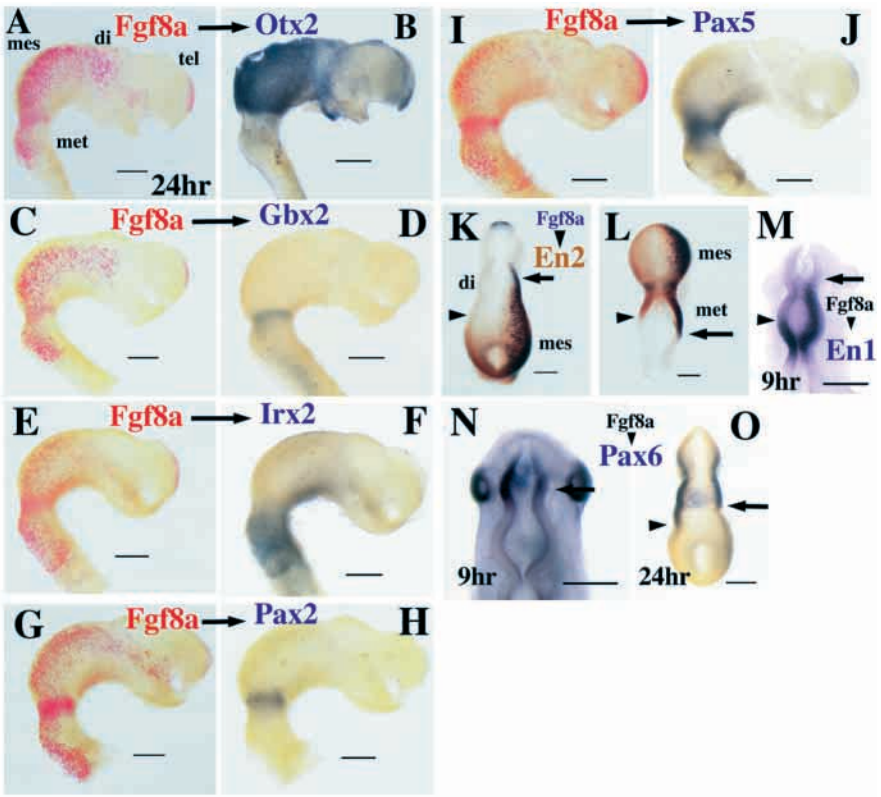

Fig. 4. Effects of Fgf8a on down stream gene expression. Fgf8a did not affect *Otx2* (A, B), *Gbx2* (C,D), *c-Irx2* (E,F), *Pax2* (G,H), or *Pax5* (I,J) expression. En 2 expression was upregulated by Fgf8a in the diencephalic region (K,L). *En1* expression was also upregulated in the diencephalic region (M). *Pax6* expression was repressed at the posterior diencephalon (N,O). Whole-mount in situ hybridization for *Fgf8a* (A,C,E,G,I, red), and for *Otx2* (B), *Gbx2* (D), *c-Irx2* (F), *Pax2* (H), *Pax5* (J), *En1* (M), and *Pax6* (N,O), all in purple. (K,L) In situ hybridization for *Fgf8a* (purple) and immunohistochemistry showing En2 expression (brown). An arrow indicates the rostral limit of En2 (K), *En1* (M), and *Pax6* (N,O) expression, and the caudal limit of En2 (L) expression on the experimental side. An arrowhead indicates the limit of corresponding gene expression on the control side (K-O). Scale bars, 600 μm.

(0.1 μg/μl) and *Otx2* (1.0 μg/μl) expression vectors recovered the tectal swelling (6/6, Fig. 7C,D). Moreover, diencephalon and metencephalon changed their fate to tectum. These results confirm that Fgf8 signal activates the gene expression cascade for the optic tectum in the neural tube only where *Otx2* is expressed. Therefore, Otx2 is a key molecule in mesencephalic determination.

DISCUSSION

We have shown here by misexpression experiments that (1) Fgf8a induced En2 expression in the diencephalon and changed its fate to the mesencephalon, (2) Fgf8b upregulated *Gbx2*, *Pax2* and *Irx2* expression, repressed *Otx2* expression, and induced cerebellum in place of the tectum and the caudal diencephalon, and (3) weak Fgf8b signals exerted Fgf8a-type effects. We have confirmed that Otx2 is involved in the competence of mesencephalon.

Classical transplantation experiments showed that presumptive diencephalon could change its fate to differentiate into tectum when transplanted into the mesencephalon near the isthmus (Nakamura et al., 1986; Nakamura and Itasaki, 1992). Then it was shown that isthmus has an organizing activity for the tectum and the cerebellum (Martinez et al., 1991; Martinez et al., 1995). Since an Fgf8-soaked bead implanted into the diencephalon induced expression of En2 and *Wnt1*, and since the neighboring prospective diencephalon differentiated into the tectum, it was assumed that Fgf8 is the isthmus organizing molecule (Crossley et al., 1996). Subsequent gain-of-function studies of Fgf8 in chick and mice, and analyses in mutant zebrafish and mice have all suggested that Fgf8 is a signaling molecule emanated from the isthmus (Brand et al., 1996; Lee et al., 1997; Liu et al., 1999; Martinez et al., 1999; Meyers et al., 1998; Reifers et al., 1998; Shamim et al., 1999).

In the present study, Fgf8a did not affect *Otx2, Gbx2 or Pax2/5* expression. Fgf8a induced *En1* and En2 expression in the diencephalon, and as a result, *En1* and En2 expression extended anteriorly to the presumptive diencephalic region. Corresponding to the anterior extension of the *En* expression domain, tectal swelling extended anteriorly. The posterior limit of the *Pax6* expression domain shifted anteriorly. As indicated by Matsunaga et al. (Matsunaga et al., 2000), the interface of *Pax6* and *En1* expression domains corresponded to the boundary between the diencephalon and the tectum on the experimental side. Since the oculomotor nucleus extended anteriorly, and since additional oculomotor nerve trunks were observed after *Fgf8a* misexpression, it was concluded that Fgf8a converted the fate of the alar plate and the basal plate from diencephalon to mesencephalon.

After *Fgf8b* misexpression, tectal tissue was completely replaced by cerebellar tissue. In this ectopic cerebellum, the external granular layer and Purkinje cell layer differentiated. Purkinje cell differentiation in the ectopic tectum was confirmed by immunocytochemistry with anti-calbindin antibody on E15.5 embryos. After *Fgf8b* misexpression *Gbx2*, *Pax2* and *Irx2* expression extended to the diencephalic level, and *Otx2* expression disappeared from the mesencephalon and the posterior diencephalon. This phenomenon suggests that the isthmus may have moved to the prospective diencephalic level. Thus the motor nucleus that differentiated at the level of original anterior diencephalon (see Fig. 3L) may be the trochlear nucleus. The effect of Fgf8b both morphologically and on downstream gene expression, indicate that the alar plate of the presumptive mesencephalon and the posterior diencephalon changed their fate to the cerebellum.

It is a very interesting phenomenon that the quite different architecture of tectum and cerebellum differentiate as a result of exposure to the isthmus organizing signal. Their mode of differentiation is also quite different. The difference in sequence between *Fgf8a* and *Fgf8b* is subtle (see Fig. 1A;

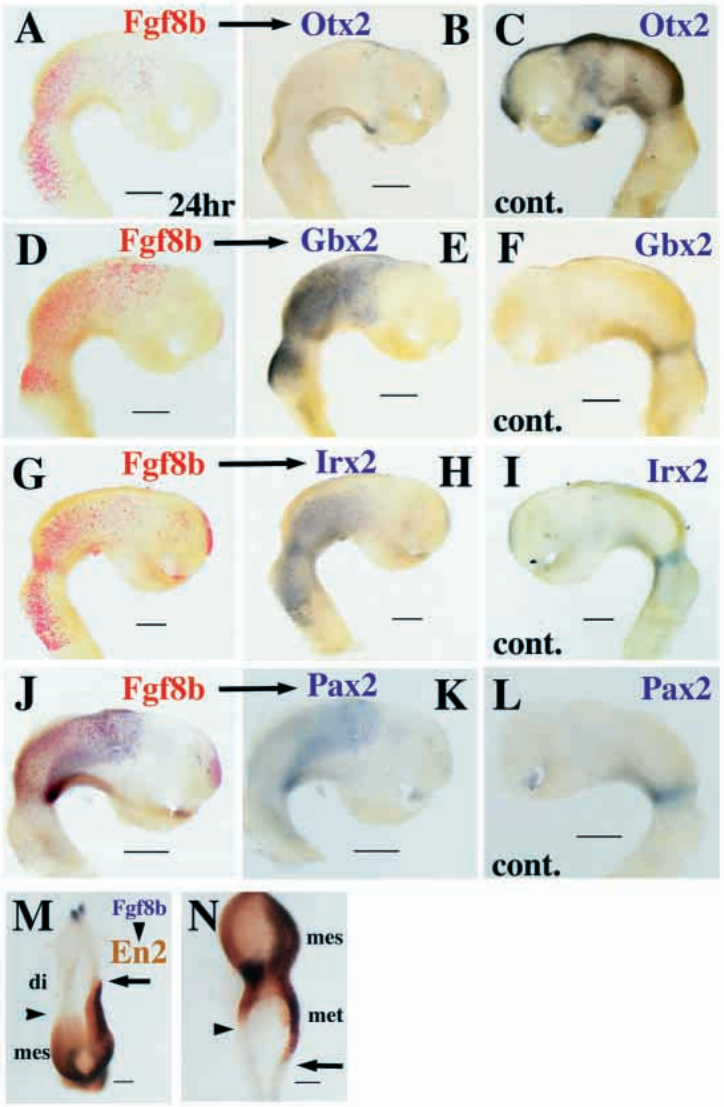

Fig. 5. Effects of Fgf8b on down stream gene expression. Fgf8b repressed *Otx2* (A-C), induced *Gbx2* (D-F), *Irx2* (G- I) and *Pax2* (J-L) expression. Fgf8b upregulated En2 expression in the diencephalic region (M,N). Whole-mount in situ hybridization for *Fgf8b* (A,D,G,J, red), and for *Otx2* (B,C), *Gbx2* (E,F), *Irx2* (H,I), *Pax*2 (K,L) in purple. (M,N) In situ hybridization for *Fgf8b* (purple) and immunohistochemistry showing En2 expression (brown), C,F,I and L show the control side. An arrow indicates the rostral (M) and caudal (N) limit of En2 expression on the experimental side, and an arrowhead indicates the rostral (M) and caudal (N) limit of En2 expression on the control side. Scale bars, 600 μm.

Crossley et al., 1995; MacArthur et al., 1995a), but the effect of misexpression of *Fgf8a* and *Fgf8b* is very different as shown in the present study and in transgenic mice in which *Fgf8a* or *Fgf8b* was misexpressed under *Wnt1* regulation (Lee et al., 1997; Liu et al., 1999). *Fgf8a*-misexpressing mice show overgrowth of the di-mesencephalic region (Lee et al., 1997, Liu et al., 1999). However, *Fgf8b*-misexpressing mice show transformation of the presumptive diencephalon and mesencephalon to metencephalon as judged by marker gene expression (Liu et al., 1999). *Fgf8b* transgenic mice showed severe exencephaly, so that the final fate of the presumptive mesencephalon and diencephalon was not reported by Liu et al. (Liu et al., 1999). The present study is the first to show that Fgf8b changed the fate of the entire mesencephalic alar plate to differentiate as cerebellum, while Fgf8a exerted little effect on the mesencephalon. It has been shown that the expression level of a transfected gene by in ovo electroporation is dependent on the concentration of the DNA solution injected (Momose et al., 1999). Since electroporation with 1% Fgf8b expression vector exerted a similar effect as Fgf8a, the type difference between Fgf8a and Fgf8b as an isthmic organizing signal can be attributed to the intensity of the signal. Liu et al. (Liu et al., 1999) also interpreted the phenotype difference between Fgf8a and Fgf8b transgenic mice as quantitative, the effect of Fgf8a being milder than that of Fgf8b. Implantation of beads soaked in Fgf8b in chick embryos also support this hypothesis (Martinez et al., 1999). Martinez et al. implanted the Fgf8b-soaked bead in the diencephalon, and showed that the cells closest to the bead acquired the cerebellar characteristics, and that the tectum was induced surrounding the cerebellar islet. In this context, Fgf8 signal might have been weak in the Fgf8b bead implantation experiment of Crossley et al. (Crossley et al., 1996) in which midbrain structure was induced in the diencephalon. The results of Shamim et al. (Shamim et al., 1999) in which Fgf8a- and Fgf8b-soaked beads implanted in the diencephalic region induced similar

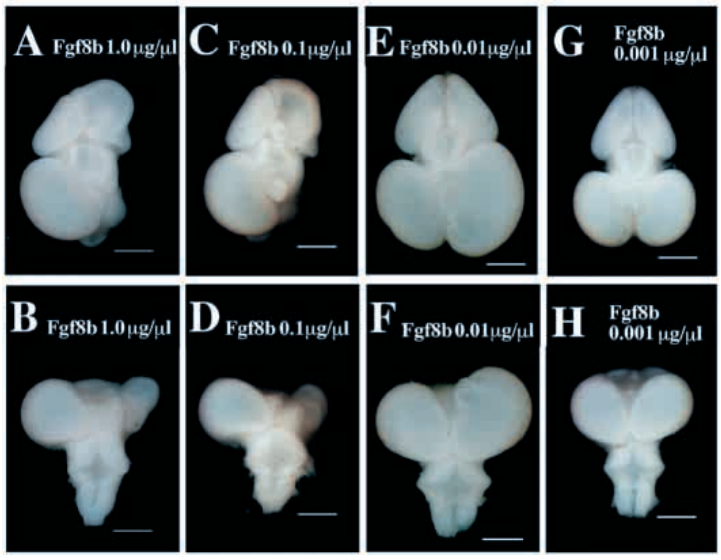

Fig. 6. Semi-quantitative analysis of *Fgf8b* misexpression. Electroporation of pMiwFgf8b at a concentration of 1.0 μg/μl (A,B) and 0.1 μg/μl (C,D) resulted in fate change of the mesencephalic alar plate to the cerebellum. However, electroporation of pMiwFgf8b at a concentration of 0.01 μg/μl resulted in enlargement of the tectum (E,F), as seen with Fgf8a. Electroporation at a concentration of 0.001 μg/μl had no effect (G,H). Embryos were all fixed at E7.5. Scale bars are 4 mm.

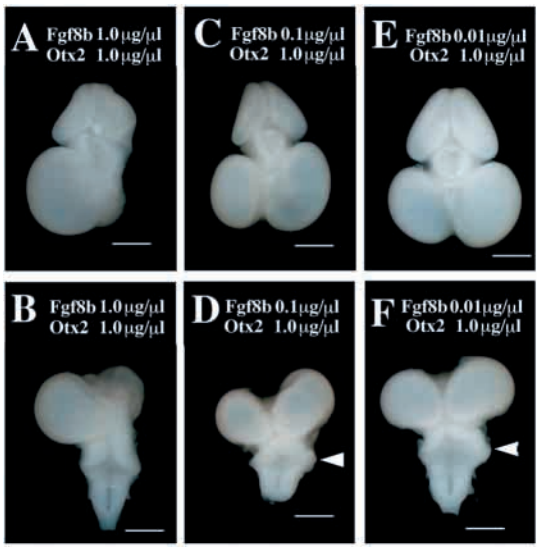

Fig. 7. Co-transfection of *Fgf8b* with *Otx2*. When *Otx2* was co-electroporated, the effect of Fgf8b was converted to an Fgf8a-type effect at a concentration of 0.1 μg/μl, 10 times higher concentration than single *Fgf8b* electroporation. Arrowhead indicates ectopic tectal structure in the metencephalon (D,F). Embryos were fixed at E7.5. Scale bars, 4 mm.

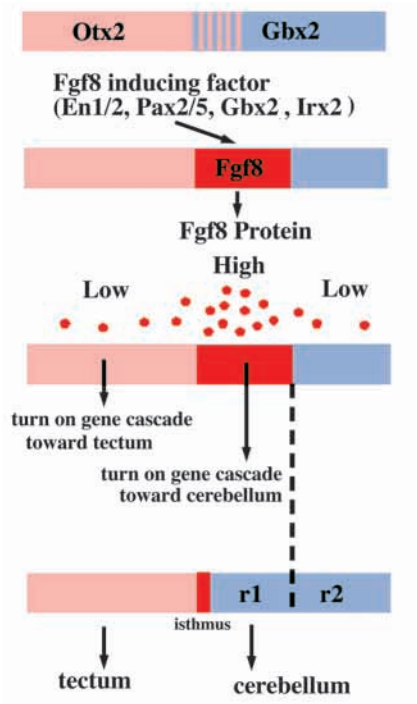

Fig. 8. Schematic drawing to show the inductive activity of Fgf8. In normal development, *Fgf8* expression is induced at the interface of *Otx2* and *Gbx2* expression domains, overlapping with *Gbx2*. *En1* and *Pax2* are expressed both in the mesencephalon and the metencephalon, and they may be involved in the initiation of *Fgf8* expression. In the metencephalic region, Fgf8 signal (red) is strong enough to support *c-Irx2* and *Gbx2* expression and to repress *Otx2* expression, which may induce this region to develop as R1. Consequently, the alar plate of R1 differentiates as cerebellum. In contrast, in the mesencephalic region, Fgf8 signal may be too weak to repress *Otx2* expression. The alar plate differentiates as tectum.

changes are consistent with our studies, in that weak Fgf8b signal exerted similar effects to Fgf8a.

Responsiveness of the tissue to the signaling molecule, may be determined by the combination of the transcription factors expressed in the responding area. It has been suggested that *Otx2* and *En1* are indispensable for the tectum development (Araki and Nakamura, 1999; Wurst et al., 1994). Otx2 misexpression in the metencephalon resulted in ectopic differentiation of the tectum or inferior colliculus in the metencephalic region (Broccoli et al., 1999; Katahira et al., 2000). Misexpression of *En2* or *En1* in the diencephalon also changed the fate of the diencephalic alar plate to that of mesencephalon (Araki and Nakamura, 1999). Since *En1* is expressed commonly in the metencephalon and in the mesencephalon, we thought that *Otx2* may be a key molecule for mesencephalic competence. As discussed above, electroporation with a lower concentration of *Fgf8b* expression vector exerted the Fgf8a-type effects. Co-transfection of *Otx2* turned the Fgf8b-type to Fgf8a-type effects at higher concentration of Fgf8b than electroporation with Fgf8b alone. The results indicate that Otx2 is crucial for the mesencephalic competence.

How do these results relate to the isthmic organizing signal and differentiation of the tectum and the cerebellum in normal development? It has been suggested that *Fgf8* expression is settled at the interface of *Otx2* and *Gbx2* expression, overlapping with *Gbx2* expression (Broccoli et al., 1999; Hidalgo-Sanchez, 1999; Millet et al., 1999; Irving and Mason; 2000; Katahira et al., 2000). The region that is exposed to the strong Fgf8 signal, and expresses *Gbx2* and *c-Irx2* may acquire characteristics of rhombomere 1 (R1) (Fig. 8). Indeed, the results of our RT-PCR show that *Fgf8b* is predominantly expressed in the isthmic region (see Fig. 1). In the R1 region, Hox genes are not expressed, and cerebellum differentiates from the dorsal part of R1, the rhombic lip (Irving and Mason, 2000; Martinez and Alvarado-Mallart, 1989; Wingate et al., 1999). *Gbx2* and *Irx2* are expressed in R2, but the fate is different. It may be because *Hoxa2* is expressed in R2 (Irving and Mason, 2000; Wingate et al., 1999). Since *Otx2* represses *Fgf8* expression (Broccoli et al., 1999; Katahira et al., 2000), *Fgf8* transcripts do not localize in the mesencephalon (Irving and Mason, 2000; Martinez et al., 1999). Although we do not know the exact range of Fgf8 signaling, diffusion of Fgf8 protein in the mesencephalon may be limited. Thus, the mesencephalic region may receive weak Fgf8 signal, which does not repress *Otx2* expression. Consequently, mesencephalic alar plate may differentiate into the tectum. Since *En*, which confers posterior characteristics to the tectum (Itasaki and Nakamura, 1996), is so sensitive to upregulation by Fgf8, Fgf8 may play a crucial role in rostrocaudal polarity formation of the tectum, as proposed by Shamim et al. (Shamim et al., 1999). The idea that the region that receives strong Fgf8 signal acquires the R1 characteristics may also be supported by the result of Fgf8b-bead implantation experiment in which cerebellar structure was induced just near the bead, and the tectum at a distance from the bead (Martinez et al., 1999). Recently, Fgf17 and Fgf18 were shown to be expressed at the mes/metencephalic boundary (Maruoka et al., 1998; Xu et al., 2000). It was also reported that expression patterns and the affinity to the receptors of Fgf8, Fgf17 and

Fgf18 in vitro are similar (Xu et al., 1999). Fgf18 has been found to induce *Fgf8* expression in the caudal diencephalon, and transform the caudal diencephalon to the midbrain (Ohuchi et al., 2000). *Fgf17* knock-out mice exhibited a tissue loss in the caudal part of the mesencephalon, inferior colliculus and the vermis cerebellum (Xu et al., 2000). These results indicate functional redundancy of these genes. Further analysis is required to assess the differential role of these genes.

En1 and En2 are so sensitive to upregulation by Fgf8 signal that both are induced by Fgf8a in the diencephalon (see Fig. 4K-M). This may well explain why Fgf8a transforms the presumptive diencephalon into the mesencephalon.

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