The Fgf8 signal causes cerebellar differentiation by activating the Ras-ERK signaling pathway

Tatsuya Sato1,2,* and Harukazu Nakamura1,2,†

1Department of Molecular Neurobiology, Graduate School of Life Sciences, Tohoku University, Seiryo-machi 4-1, Aoba-ku, Sendai 980-8575, Japan
2Institute of Development, Aging and Cancer, Tohoku University, Seiryo-machi 4-1, Aoba-ku, Sendai 980-8575, Japan
*Present address: Skirball Institute of Biomolecular Medicine, New York University School of Medicine, 540 First Avenue, New York, NY 10016, USA
†Author for correspondence (e-mail: nakamura@idac.tohoku.ac.jp)

Summary

The mes/metencephalic boundary (isthmus) is an organizing center for the optic tectum and cerebellum. Fgf8 is accepted as a crucial organizing signal. Previously, we reported that Fgf8b could induce cerebellum in the mesencephalon, while Fgf8a transformed the presumptive diencephalon into mesencephalon. Since lower doses of Fgf8b exerted similar effects to those of Fgf8a, the type difference could be attributed to the difference in the strength of the signal. It is of great interest to uncover mechanisms of signal transduction pathways downstream of the Fgf8 signal in tectal and cerebellar development, and in this report we have concentrated on the Ras-ERK pathway. In normal embryos, extracellular-signal-regulated kinase (ERK) is activated at the site where Fgf8 mRNA is expressed. Fgf8b activated ERK while Fgf8a or a lower dose of Fgf8b did not activate ERK in the mes/metencephalon. Disruption of the Ras-ERK signaling pathway by a dominant negative form of Ras (RasS17N) changed the fate of the metencephalic alar plate from cerebellum to tectum. RasS17N canceled the effects of Fgf8b, while co-transfection of Fgf8a and RasS17N exerted additive effects. Disruption of Fgf8b, not Fgf8a, by siRNA resulted in posterior extension of the Otx2 expression domain. Our results indicate that the presumptive metencephalon receives a strong Fgf8 signal that activates the Ras-ERK pathway and differentiates into the cerebellum.

Key words: Tectum, Cerebellum, Fgf8, Isthmus, Cell signaling, Ras, ERK, Chick

Introduction

Classical transplantation studies in chick embryos first revealed that the mes/metencephalic boundary (MHB, also referred to as the isthmus) has an organizing activity for the mesencephalon and the metencephalon (reviewed by Joyner et al., 2000; Simeone, 2000; Nakamura, 2001; Martinez, 2001; Wurst and Bally-Cuif, 2001; Rhinn and Brand, 2001). Presumptive diencephalon transplanted near the isthmus changed its fate to the tectum (Nakamura et al., 1986; Nakamura and Itasaki, 1992), and the isthmic region transplanted into the diencephalon and rhombencephalon induced tectum and cerebellum, respectively (Martinez et al., 1991; Martinez et al., 1995). Fgf8-soaked beads are able to mimic the isthmus, indicating that Fgf8 is a crucial isthmic-organizing molecule for the mesencephalon (Crossley et al., 1996; Martinez et al., 1999; Shamim et al., 1999). This notion is supported by genetic studies of Fgf8 mutant mice and zebrafish, in which the mes/metencephalic development is disrupted (Meyers et al., 1998; Reifers et al., 1998).

There are eight Fgf8 isoforms identified so far (Crossley and Martin, 1995; MacArthur et al., 1995b). Among these, Fgf8a and Fgf8b are expressed in the chick isthmus (Sato et al., 2001). Fgf8a and Fgf8b have different organizing activities in vivo. Transgenic mice in which Fgf8a was misexpressed under the control of a Wnt1 enhancer showed overgrowth of the di-
tube using an anti-di-phosphorylated ERK antibody, and found that ERK was activated strongly in Fgf8 mRNA-expressing regions. We also found that Fgf8b could activate ERK more strongly than Fgf8a in the mes/metencephalon. Misexpression of a dominant-negative form of Ras (RasS17N) was carried out to disrupt the Ras-ERK pathway. RasS17N changed the fate of the metencephalic alar plate from cerebellum to tectum. Application of siRNA against Fgf8b by electroporation resulted in posterior extension of the Otx2 expression domain. We propose that a strong Fgf8 signal activates the Ras-ERK pathway and ultimately results in cerebellar differentiation.

Materials and methods

Probes, cDNAs and expression vectors

cDNAs for En1, Fgf8, Pax2/5 and Gbx2 were cloned as described previously (Araki and Nakamura, 1999; Itasaki and Nakamura, 1996; Sato et al., 2001; Okafuji et al., 1999; Funahashi et al., 1999; Katahira et al., 2000). cDNAs for Otx2 and Wnt1 were kind gifts of Drs Kitamura and Wassef, respectively. An amino-terminal HA tagged dominant-negative form of Ras was obtained by the following procedures: the pCMV-RasN17 vector (Clontech) was digested with EcoRI and BamHI, then the fragments were subcloned into the pBluescript II SK(−) vector. Next, PCR was performed using this DNA construct as a template. The sequences of primers were: 5′-CATGGATCCATGGAAGTCACGCTCCCAGACTCGAGAATGACGGAATATATAAAGCTGG-3′ and 5′-AATTAACCCCTACTAAAAGGG-3′ (T7 primer). PCR products were subcloned into the pBluescript vector and sequenced. Fgf8a, Fgf8b and HA-RasS17N cDNAs were inserted into the pMiwIII expression vector (Araki and Nakamura, 1999); a derivative of pMiwSV, which has the chick–actin promoter and RSV enhancer (Suemori et al., 1990; Wakamatsu et al., 1991). In-ovo electroporation

In-ovo electroporation was carried out as described previously (Funahashi et al., 1999; Nakamura et al., 2000; Nakamura and Funahashi, 2001). Briefly, fertilized chicken embryos were incubated in humid conditions at 38°C for 30-36 hours to reach 7-10-somite stages, corresponding to stage 9-10 (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 at a distance of 4 mm, then a rectangular pulse of 25 V, 50 ms was charged four times by the electroporator (CUTY21, Tokiwa Science, Fukuoka, Japan). To monitor the ectopic expression, the GFP expression vector (pCA-GAP-GFP) (Niwa et al., 1991; Moriyoshi et al., 1996) was mixed in the DNA solution (0.35 μg/μl). Since DNA is negatively charged, only the anode side of the neural tube is transfected. The other side is used as a control.

siRNA to specifically silence Fgf8a and Fgf8b

Recently, it was shown that siRNA could specifically disrupt target mRNA by introducing siRNA expression vector (Kawahara and Nakamura, 2003). Since the difference between Fgf8a and Fgf8b is only the presence of 33 bases in Fgf8b, target sequence specific for Fgf8a and Fgf8b siRNA is limited, and was determined as shown in Fig. 7A. The 19-mer sense and antisense siRNA sequences were linked with nine nucleotide spacer (TTCAGAGA) as a loop, and six T and A bases were added as the terminal signal to the 3′ end of the forward oligonucleotides, and 5′ end of the reverse oligonucleotides, respectively. EcoRI and Apal restriction site was added to the 5′ and 3′ end of the reverse oligonucleotides, respectively. The forward and reverse oligonucleotides were annealed, and inserted into the pSilencer 1.0-U6 (Ambion).

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). RNA probes were labeled with digoxigenin (DIG) according to the manufacturer’s protocol (Promega). Alkaline phosphatase (AP)-conjugated anti-DIG antibody (Roche) was colored with nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP).

Immunohistochemistry

For whole-mount immunohistochemistry, the following monoclonal antibodies were used as primary antibodies: anti-En2 antibody, 4D9 (American Type Culture Collection), anti-di-phosphorylated ERK (Sigma), anti-neurofilament antibody, 3A10 (Developmental Studies Hybridoma Bank) and anti-HA antibody (Boehringer). For detection of En2 and activated ERK, horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Jackson) and biotinylated anti-mouse IgG were used as secondary antibodies, respectively. For detection of neurofilament, Cy3-conjugated anti-mouse IgG (Jackson) was used as a secondary antibody. For detection of HA-tag, HRP-conjugated anti-rat IgG was used. Immunoreactivity for activated ERK was detected using the ABC-Elite system (Vector Laboratories). DAB (3,3-diaminobenzidine) was adopted as the chromogen for HRP.

Histology

Embryos were fixed in 4% paraformaldehyde and embedded in HistoResin (Leica). Serial sections at 5 μm were stained with hematoxylin and eosin.

Results

ERK activation in the neural tube

One of the main signal transduction pathways downstream of the RTK is the Ras-ERK system. We first determined whether ERK is activated in the isthmus. The activated form of ERK is generated by phosphorylation of both threonine and tyrosine residues that lie adjacent to each other in the ERK sequence, the Ras-ERK system. We first determined whether ERK is activated in the neural tube

Beak implantation

AG1-X2 ion-exchange resin beads (BioRad) were washed with DMSO three times, then incubated for 20 minutes with 10 mM SU5402 (Calbiochem) in DMSO. An SU5402-soaked bead was implanted in the isthmus. At 1 or 2 hours after implantation, embryos were fixed with 4% paraformaldehyde/PBS.
misexpression (the site of ERK activation coincided with that of observed only in the diencephalon (metencephalon (Fig. 2E), ectopic activation of ERK was was transfected into diencephalon, mesencephalon and molecular marker expression (Sato et al., 2001). Misexpression both in terms of morphological effects and of specific alar plate to the tectum (Fig. 2D).

We then proceeded to test whether Fgf signaling could truly activate ERK. For this purpose, misexpression of Fgf8a and Fgf8b on the right side of the brain vesicles was carried out. As reported previously (Sato et al., 2001), Fgf8b instructs the mesencephalic alar plate to differentiate into the cerebellum (Fig. 2B,C), while Fgf8a changes the fate of the diencephalic alar plate to the tectum (Fig. 2D). Fgf8a and Fgf8b exerted different effects on ERK activation. When Fgf8a was transfected into diencephalon, mesencephalon and metencephalon (Fig. 2E), ectopic activation of ERK was observed only in the diencephalon (n=7/9) (Fig. 2F). However, the site of ERK activation coincided with that of Fgf8b misexpression (n=9/9) (Fig. 2G). We have previously reported that a weaker Fgf8b signal exerted similar effects to Fgf8a, both in terms of morphological effects and of specific molecular marker expression (Sato et al., 2001). Misexpression of Fgf8b at a concentration (0.01 μg/μl) that exerts Fgf8a-type effects resulted in activation of ERK only in the diencephalon, as was seen in the misexpression of Fgf8a (n=6/9) (Fig. 2H).

**Misexpression of a dominant-negative form of Ras causes differentiation of the tectum instead of the cerebellum**

Since ERK was activated by Fgf8b, we assumed that the Ras-ERK signaling pathway plays a pivotal role in mes/metencephalic development. In order to check this assumption, we misexpressed a dominant-negative form of Ras (RasS17N), which was shown to disrupt the Ras-ERK pathway (Feig and Cooper, 1988). Indeed, the activation level of ERK was decreased after misexpression of RasS17N by in-ovo electroporation in 7-10-somite stage embryos (n=8/11) (Fig. 3A). Misexpression of RasS17N exerted drastic effects on the development of the presumptive metencephalon (Fig. 3B-D). At E10.5 (HH36-37), a large swelling with a smooth surface was observed in the metencephalic region on the experimental side (n=7/7) (Fig. 3B,D). On the control side, the swelling displayed typical fissures of the cerebellum (Fig. 3B,C), and had an external granular layer (egl), which is also characteristic of the cerebellum at E10.5 (Fig. 3E,F). On the experimental side of the metencephalic region, the swelling lacked the external granular layer (Fig. 3E,G). Instead, the swelling had a structure similar to that of the tectum. The proper tectum at E10.5 has ten layers in addition to the neuroepithelium (Fig. 3H). In the structure on the experimental side of the metencephalic region (Fig. 3I), we could discern nine of the layers characteristic of the tectum. The outermost layer x, which is composed of optic fibers, could not be discerned. Layer x was not differentiated, possibly because all the optic fibers had already projected to the proper tectum and hence could not reach the ectopic tectum. These results indicate that Ras signaling is needed for differentiation of the cerebellum, and that disruption of Ras signaling converts the fate of the metencephalic alar plate to differentiate to the tectum. The posterior part of the swelling at the experimental side consisted of cerebellar structure.

We then examined the fate of the basal plate of the RasS17N-misexpressing metencephalon. The oculomotor and trochlear nerves are landmarks of the ventral mesencephalon and isthmus, respectively. Whole-mount immunohistochemistry with anti-neurofilament antibody revealed that the oculomotor nerve trunk originates from the ventral mesencephalon of the control side and runs toward the external ocular muscles (Fig. 3I, III). Trochlear nerve fibers arise from the nucleus, and run dorsally along the MHB to decussate at the dorsal midline (Fig. 3I, IV). In RasS17N-transfected embryos, swellings were discerned in the metencephalon (n=7/12). In the example shown in Fig. 3J,K, a rather large swelling and a smaller, more caudal swelling were discerned (Fig. 3J, arrow and arrowhead). In this embryo, very few nerve fibers were discerned at the proper trochlear nerve site. Main trochlear nerve fibers arose from the posterior portion of the large ectopic swelling (Fig. 3J, arrowheads). Nerve fibers also arose from the posterior portion of the small swelling (Fig. 3J, arrow), and these fibers merged into a main bundle while running dorsally. In some cases, small swellings differentiated in the metencephalic region (Fig. 3N), and nerve fibers arose from several points and ran dorsally (Fig. 3M). This kind of phenotype may be obtained when transfection of RasS17N was not enough to
change the fate of the entire metencephalon. These results suggest that an additional isthmus was formed caudal to the ectopic swelling due to the misexpression of Ras S17N. In another case, a nerve trunk originated from the ventral metencephalon and ran a similar course to the oculomotor nerve (Fig. 3L, arrowheads, n=4/7). Thus, we hypothesize that disruption of the Ras signal converts the fate of both the alar and basal plates of the metencephalon to that of the mesencephalon.

**Alteration of gene expression by disruption of Ras signaling**

We examined the effects of the misexpression of RasS17N on molecular markers for the mesencephalon and the metencephalon. In normal embryos, the homeobox genes, Otx2 and Gbx2, are expressed in the mesencephalon and the metencephalon, respectively (Simeone et al., 1992; Bally-Cuif et al., 1995; Millet et al., 1996; Bouillet et al., 1995; Niss and Leutz, 1998; Shamim and Mason, 1998; Hidalgo-Sanchez et al., 1999). It has been suggested that repressive interaction between Otx2 and Gbx2 determines the MHB, and that Fgf8 mRNA is induced at the interface of Otx2 and Gbx2 expression overlapping with Gbx2 expression (Millet et al., 1999; Broccoli et al., 1999; Katahira et al., 2000; Li and Joyner, 2001; Ye et al., 2001). At 24 hours after electroporation of RasS17N (E2.5, HH17), induction of Otx2 and repression of Gbx2 in the metencephalon were observed (Otx2, n=9/9, Gbx2, n=7/8) (Fig. 4A-F). Expression of Fgf8 was also repressed by RasS17N, but was induced in the caudal part of its expression belt so that the Fgf8 expression belt became wider (n=3/3) (Fig. 4G-I).

At 42 hours after electroporation (E3.25), boundary of patchy expression of Otx2 in the metencephalic region became blurred, and the Otx2-free area became narrower (n=11/13) (Fig. 4J,K). However, repression of Gbx2 just posterior to the proper mesencephalon became wider (Fig. 4L-N). Corresponding to the change of the manner of Gbx2 repression, the wide Fgf8-free region appeared just caudal to the proper mesencephalon (n=4/4). One or two Fgf8 expression line(s) appeared caudal to the Fgf8-free region (n=4/4) (Fig. 4O-Q). If we consider that Otx2 is induced in the RasS17N-expressing site, Fgf8 may have been induced at the border of the Otx2 and Gbx2 expression domains (Broccoli et al., 1999; Millet et al., 1999; Katahira et al., 2000; Ye et al., 2001).

Next, we examined the effect on Wnt1 expression. At E2.5, Wnt1 was expressed in the dorsal midline of the mesencephalon and in the caudal mesencephalon. Since the dorsal midline of the metencephalon does not express Wnt1, it is a good marker to discriminate between metencephalon and mesencephalon (Fig. 5A, control side). After disruption of Ras signaling, Wnt1 was induced in the dorsal metencephalon on the experimental side (n=3/4) (Fig. 5A,B). These effects of RasS17N on marker gene expression also support the notion that disruption of Ras signaling changes the fate of the metencephalon to that of the mesencephalon.

Effects of the misexpression of RasS17N on Pax2/5, En1/2 expression were also examined. These molecules and Fgf8 are shown to be in a positive feedback loop for their expression. This feedback loop may help to maintain the organizing activity of the isthmus (reviewed by Nakamura, 2001). Expression of Pax2/5 and En1/2 was repressed in the area where RasS17N was misexpressed (Pax2, n=3/3; Pax5, n=4/4; En1, n=4/4; En2, n=4/4) (Fig. 5C-V), suggesting that the Ras signaling pathway is necessary for maintenance of expression of these molecules in the mes/metencephalic region.

**The Ras signaling pathway functions downstream of Fgf8b, but not Fgf8a**

Morphological and gene expression analyses indicate that the Ras signaling pathway plays an important role in mes/metencephalic fate determination. To ascertain if the Ras signaling pathway functions at the downstream of the Fgf8b signal, we carried out co-transfection of Fgf8b with RasS17N. If Ras functions at the downstream of the Fgf8b signal, co-transfection of RasS17N with Fgf8b may cancel the effects of

---

**Fig. 2.** ERK activation by Fgf8 signal. (A) Immunohistochemistry with anti-dpERK 1 hour after insertion of an SU5402-soaked bead (asterisk). ERK activation is repressed by SU5402, an inhibitor of the Fgf receptor. (B) Dorsal and (C) lateral view of an E14.5 brain after misexpression of Fgf8b. Instead of the tectum, cerebellum has differentiated in the mesencephalic region (arrow). (D) Dorsal view of an E6.5 brain after misexpression of Fgf8a. The tectum enlarged because the fate of the diencephalic alar plate was changed to tectum. (E) Misexpression of GFP at 3 hours after electroporation. The GFP misexpression site corresponds to that of Fgf8 shown in (F-H). (F-H) Immunohistochemistry with anti-dpERK antibody after misexpression of Fgf8a 1 µg/µl (F), Fgf8b 1 µg/µl (G), Fgf8b 0.01 µg/µl (H). ERK was activated only in the diencephalon by Fgf8a (F). ERK was activated at the site where Fgf8b was misexpressed through the diencephalon, mesencephalon and metencephalon (G). Misexpression of Fgf8b at a concentration of 0.01 µg/µl caused ERK activation only in the diencephalon (H), as was the case of Fgf8a misexpression. di, diencephalon; mes, mesencephalon; met, metencephalon; tel, telencephalon; tect, tectum; cer, cerebellum; cer-ect, ectopic cerebellum. Scale bars: 200 µm (A,F-H), 4 mm (B-D).
Fgf8b misexpression. Conversely, if Ras does not transduce the Fgf8b signal, co-transfection may exert additive effects. After co-transfection, some large swellings were observed on the experimental side of the mes/metencephalon of E10.5 embryos (m=4/4) (Fig. 6A,B). Histologically, these swellings showed a tectal structure (compare Fig. 6C,D with 6E,F), in agreement with our prediction. The anterior part of the presumptive metencephalon differentiated into the tectum (Fig. 6A,E). In the posterior part of the presumptive metencephalon, target genes of ERK may have been already turned prior to the posterior part of the presumptive metencephalon to the mesencephalon. These results suggest that the Fgf8b signal activates the Ras-ERK signal pathway to organize the metencephalic differentiation. To confirm this notion, we tried differential disruption of Fgf8a and Fgf8b by the siRNA method. Since the vector-based-siRNA method has been realized recently (Katahira and Nakamura, 2003), we introduced the siRNA expression vectors to the metencephalon and mesencephalon by electroporation (Fig. 7A).

Co-transfection of Fgf8a and RasS17N exerted additive effects (m=4/4), whereby there was differentiation of the ectopic tectum in the diencephalon (Fig. 6G,H) and in the metencephalon (Fig. 6I). This data suggest that activation of Ras signaling is necessary for cerebellar development, but is not crucial for tectal differentiation.

**Differential silencing of Fgf8a and Fgf8b by siRNA method**

We previously showed that Fgf8b could change the fate of the mesencephalon to the metencephalon (Sato et al., 2001), and have shown in the present study that disruption of the Ras-ERK signaling pathway resulted in the fate change of the metencephalon to the mesencephalon. These results suggest that the Fgf8b signal activates the Ras-ERK signal pathway to organize the metencephalic differentiation. To confirm this notion, we tried differential disruption of Fgf8a and Fgf8b by the siRNA method. Since the vector-based-siRNA method has been realized recently (Katahira and Nakamura, 2003), we introduced the siRNA expression vectors to the metencephalon and mesencephalon by electroporation (Fig. 7A).

Massive degradation of Fgf8 mRNA could not be detected after Fgf8b-siRNA application, but downregulation of Fgf8 mRNA to some extent could be detected (m=5/7) (Fig. 7B-E). Degradation of Fgf8 mRNA by Fgf8a-siRNA could not be detected (m=8/8) (Fig. 7F-I). Efficient degradation of Fgf8 mRNA could be observed after application of a mixture of Fgf8a- and Fgf8b-siRNA (m=3/5) (Fig. 7J-M). Since the Fgf8 probe hybridizes to both Fgf8a and Fgf8b mRNA, disruption of each Fgf8 mRNA after application of each siRNA may be more than we could observe.

Next we checked the effects of differential disruption of Fgf8a and Fgf8b by siRNA on ERK activation. The activation level of ERK was decreased after electroporation of Fgf8b-siRNA (m=7/10) (Fig. 7N) and of both Fgf8a- and Fgf8b-siRNA (m=7/11) (Fig. 7P). Fgf8a-siRNA alone did not affect
the activation level of ERK (n=11/14) (Fig. 7O). RasS17N more effectively repressed ERK activation than Fgf8b-siRNA (compare Fig. 3A with Fig. 7N). The difference may be due to the fact that Fgf8 exerts its effects non-cell autonomously but that RasS17N exerts its effects cell autonomously. If Fgf8 mRNA is degraded by siRNA in some cells, the Fgf8 signal from the adjacent intact cells may take its place. However, RasS17N shuts off the downstream Ras signal of the transfected cell.

The effects of siRNA on Otx2 expression were examined, since Otx2 misexpression in the metencephalon changes its fate to the mesencephalon (Katahira et al., 2000). Transfection of Fgf8b-siRNA resulted in induction of Otx2 expression in the isthmus (n=4/14) (Fig. 7Q-S); that is, the Otx2 expression domain extended caudally, although the effect is very subtle because of the above-mentioned reason. Transfection of Fgf8a-siRNA did not affect Otx2 expression (n=9/9) (Fig. 7T-V). The effect of Fgf8b-siRNA on Otx2 expression also suggests that disruption of Fgf8 mRNA may have occurred more than we could detect.

Discussion

Our study has demonstrated that: (1) ERK was activated at sites of Fgf8 mRNA expression; (2) misexpression of Fgf8b activated ERK and induced ectopic cerebellum in the mesencephalon; (3) misexpression of Fgf8a or a lower dose of Fgf8b activated ERK only in the diencephalon, where ectopic tectum differentiated; (4) disruption of Ras signaling by a dominant-negative form of Ras changed the fate of the metencephalic alar plate from cerebellar to tectal development; (5) co-electroporation of a dominant-negative form of Ras with Fgf8b canceled the Fgf8b effects, while co-electroporation with Fgf8a exerted additive effects; and (6) distinct disruption of Fgf8b by siRNA resulted in repression of ERK activity, and in a caudal shift of the Otx2 expression domain.

Eight isoforms of Fgf8 have been identified to date (Crossley and Martin, 1995; MacArthur et al., 1995b), with Fgf8a and Fgf8b being expressed in the isthmus (Sato et al., 2001). Fgf8a and Fgf8b possess different organizing activities for brain development. Fgf8b-soaked beads implanted in the presumptive diencephalon induce cerebellar structures closest...
to the bead with tectum around the mini cerebellum (Martinez et al., 1999). Transgenic mice in which Fgf8b was misexpressed under the control of a Wnt1 enhancer changed the property of the presumptive diencephalon and mesencephalon to that of the metencephalon (Liu et al., 1999). Moreover, misexpression of Fgf8b by electroporation completely changed the fate of the mesencephalic alar plate so that it differentiated into cerebellum (Sato et al., 2001; Liu et al., 2003). Misexpression of Fgf8a caused expansion of the midbrain (Lee et al., 1997; Sato et al., 2001). Although Fgf8a and Fgf8b show different organizing activities, lower doses of Fgf8b exert similar effects to those of Fgf8a; the tectum was induced around the mini cerebellum in Fgf8b-bead implantation experiments, and electroporation with lower doses of Fgf8b exerted similar effects to those seen with Fgf8a (Sato et al., 2001; Liu et al., 2003). These results suggest that the difference in organizing activity between Fgf8a and Fgf8b is attributable to the difference in the intensity of the signal. This notion was further confirmed by the results of this study. Misexpression of Fgf8b at 1 μg/μl resulted in activation of ERK at Fgf8b misexpressing sites throughout the diencephalon and metencephalon, while misexpression of Fgf8b at 0.01 μg/μl or Fgf8a at 1 μg/μl resulted in activation of ERK in only a portion of the diencephalon.

Ras, a member of a group of small GTP-binding proteins, is activated downstream of various RTKs and activates Raf, Mek and ERK in turn (reviewed by Katz and McCormick, 1997; Rommel and Hafen, 1998). Since ERK was activated in the isthmus and sites of Fgf8b misexpression, we assumed that activation of the Ras-ERK pathway is necessary for metencephalic fate determination. Fate change of the alar plate is easily identifiable because of the distinct structures of the tectum and cerebellum. As expected, disruption of the Ras-ERK pathway by a dominant-negative form of Ras (RasS17N) in the alar plate of the metencephalon caused its fate change to the tectum. To follow the development of the basal plate, we paid attention to the oculomotor and trochlear nerves. In RasS17N-transfected embryos, nerve fibers running a similar course to that of the trochlear nerve arose from the caudal end of the ectopic swelling(s) in the metencephalic region. Additional nerve trunks similar to the oculomotor nerve originated from the ventral metencephalon in some cases. Moreover, at 24 hours after electroporation of RasS17N, induction of Otx2 and repression of Gbx2 in the metencephalon occurred. Thus, we concluded that disruption of Ras signaling by a dominant-negative form of Ras converted the fate of the presumptive metencephalon to that of the mesencephalon. As in the case of Otx2 misexpression (Katahira et al., 2000), Otx2 induction was patchy at first, but a large tectum differentiated in the most effective case. Repression of Gbx2 and Fgf8 was also patchy at first (24 hours after electroporation), but a wide region in which Gbx2 and Fgf8 were not expressed appeared just posterior to the proper mesencephalon (42 hours after electroporation). Fgf8 expression line(s) were established posterior to the Fgf8-free region. The results indicate that regulation of Otx2, Gbx2 and Fgf8 expression may have taken place. Thus new Fgf8 line(s) may have served as a new organizer, and most of the presumptive r1 region may have changed its property to that of the mesencephalon. In some cases, fate change to mesencephalon may have occurred patchily because a number of trochlear nerves differentiated in the metencephalic region (see Fig. 3M,N).

Further evidence to support the hypothesis that Ras signaling plays an important role in metencephalic fate determination is provided by the results of this study. The results show that disruption of Ras signaling by a dominant-negative form of Ras converted the fate of the presumptive metencephalon to that of the mesencephalon. As expected, disruption of Ras signaling by a dominant-negative form of Ras converted the fate of the presumptive metencephalon to that of the mesencephalon. As in the case of Otx2 misexpression (Katahira et al., 2000), Otx2 induction was patchy at first, but a large tectum differentiated in the most effective case. Repression of Gbx2 and Fgf8 was also patchy at first (24 hours after electroporation), but a wide region in which Gbx2 and Fgf8 were not expressed appeared just posterior to the proper mesencephalon (42 hours after electroporation). Fgf8 expression line(s) were established posterior to the Fgf8-free region. The results indicate that regulation of Otx2, Gbx2 and Fgf8 expression may have taken place. Thus new Fgf8 line(s) may have served as a new organizer, and most of the presumptive r1 region may have changed its property to that of the mesencephalon. In some cases, fate change to mesencephalon may have occurred patchily because a number of trochlear nerves differentiated in the metencephalic region (see Fig. 3M,N).

Fig. 5. Effects of RasS17N misexpression on Wnt1, Pax2, Pax5, En1, En2 and Fgf8. Effects on Wnt1 (A,B), Pax2 (C-G), Pax5 (H-L), En1 (M-Q), En2 (R-V). Brown, immunohistochemical staining against HA-tag. Blue, signal for in-situ hybridization. Dorsal view (A,B), Left (control) side of the brain vesicles (C,H,M,R), right (transfected) side (D,E,I,J,N,O,S,T). Higher magnification (F,G,K,L,P,Q,U,V). The areas enclosed by the dashed line on (E,J,O,T) corresponds to (G,L,Q,V), respectively. Wnt1 was induced in the dorsal metencephalon (arrowheads on A). Expression of Pax2/5 and En1/2 were repressed by RasS17N misexpression. Scale bars: 200 μm.
the Ras-ERK pathway is activated by Fgf8b to result in metencephalic differentiation comes from co-transfection studies with RasS17N and Fgf8b. If the Ras-ERK pathway does indeed transduce the Fgf8 signal, then co-transfection may cancel the Fgf8 signal. However, if the Ras-ERK pathway does not transduce the Fgf8 signal, co-transfection may exert additive effects. Accordingly, co-transfection of RasS17N and Fgf8b canceled the effects of Fgf8b misexpression, while co-transfection of Fgf8a and RasS17N caused differentiation of the ectopic tectum in the diencephalon and in the metencephalon, displaying the additive effects of Fgf8a and RasS17N misexpression. Distinct disruption of Fgf8a and Fgf8b also supports the notion that Fgf8b activates Ras-ERK signaling pathway to organize cerebellar differentiation. Disruption of Fgf8b by its specific siRNA resulted in a decrease in the activation level of Erk, and in caudal extension of the Otx2 expression domain. siRNA for Fgf8a did not affect the activity of Erk. In conclusion, the results indicate that Fgf8b functions as the organizer for the metencephalon by activating the Ras-ERK pathway.

Since Fgf8 mutant mice or zebrafish show disruption of the mes/metencephalon determination of the mesencephalon. To accord our assumption, animal cap assay indicated that the PLCγ signaling pathway through Fgf receptor IV (FgfR4) is responsible for the fate decision of the mesencephalon (Umbhauer et al., 2000). However, it was suggested that FgfR1 is the receptor for the Fgf8 signal in the isthmus region (Liu et al., 2003; Trokovic et al., 2003). So far, it is not reported that FgfR4 is expressed in the isthmus region as suggested (Walshe and Mason, 2000). Further study is needed to determine what signaling pathway is responsible for the mesencephalic determination.

In the mesencephalon, the Fgf8-Ras-ERK signaling pathway may be involved in rostrocaudal polarity formation. For the rostrocaudal polarity formation, it is suggested that En confers caudal property to the tectal anlage so that the rostrocaudal polarity of the tectum is determined according to a gradient of En (Itasaki and Nakamura, 1996; Friedman and O’Leary, 1996). ERK is activated in a gradient in the mesencephalon, as revealed by whole-mount immunohistochemistry. The gradient corresponds to that of En2 expression at the 14-somite stage, when the rostrocaudal axis is still plastic. Pax2/5, En1/2 and Fgf8 act in a positive feedback loop (reviewed by Nakamura, 2001). Disruption of Ras signaling caused repression of Pax2/5 and En1/2 expression. Thus, the Ras-ERK pathway, which is activated by Fgf8, may play a crucial role in formation of the rostrocaudal polarity of the tectum.

In normal embryos around the 8-somite stage, ERK was activated in the region where Fgf8 mRNA was expressed. In the metencephalon, ERK became inactivated by the 14-somite stage, while it remained activated in the mesencephalon. This
indicates that the gene expression cascade favoring cerebellar differentiation has proceeded by the 10-somite stage, meaning that the fate of the metencephalon is determined by this time. This notion is supported by ectopic transplantation studies that show that while the rostrocaudal axis of the mesencephalon is not fixed at the 10-somite stage, the fate of the mesencephalon and metencephalon is already determined (Nakamura et al., 1986; Nakamura et al., 1988; Ichijo et al., 1990; Matsuno et al., 1990).

Focusing on mechanisms of mes/metencephalic development (Fig. 8), expression of \( Fgf8 \) mRNA is induced at the interface of \( Otx2 \) and \( Gbx2 \) expression, overlapping with \( Gbx2 \) expression; that is, at the presumptive metencephalon (Millet et al., 1999; Broccoli et al., 1999; Hidalgo-Sanchez et al., 1999; Ye et al., 2001; Garda et al., 2001; Li and Joyner, 2001; Martinez-Barbera et al., 2001; Li et al., 2002). Consequently, the presumptive metencephalic region may receive a strong \( Fgf8 \) signal, in turn activating the Ras-ERK pathway, which may result in turning on the gene cascade favoring development of the cerebellum. The cascade might be turned on before the 10-somite stage, because ERK becomes strongly activated at around the 8-somite stage, its activity gradually weakening thereafter. Our results correspond well to the classical transplantation experiments that show that the fate of the mesencephalon and metencephalon is determined before the 10-somite stage. In the mesencephalon, the gene expression cascade toward cerebellar differentiation may not be turned on.

Fig. 7. Distinct disruption of \( Fgf8a \) and \( Fgf8b \) by siRNA. (A) Alignment of partial sequence of \( Fgf8a \) and \( Fgf8b \) mRNA. The target sequence of \( Fgf8a- \) and \( Fgf8b \)-siRNA is underlined with red and green, respectively. The number indicates the number from the start codon. (B-E) Effects of \( Fgf8b \)-siRNA on \( Fgf8 \) expression. We could detect disruption of \( Fgf8 \) mRNA to some extent. Since the probe used for in-situ hybridization hybridized to both \( Fgf8a \) and \( Fgf8b \), disruption of \( Fgf8b \) may be more than we can detect. (F-I) Effects of \( Fgf8a \)-siRNA on \( Fgf8 \) expression. We could not detect the effect of siRNA. This may be due to the fact that \( Fgf8b \) is predominantly expressed in the isthmus. (J-M) Application of both \( Fgf8a \)- and \( Fgf8b \)-siRNA. Electroporation of both \( Fgf8a \)- and \( Fgf8b \)-siRNA resulted in distinct reduction of \( Fgf8 \) mRNA in the isthmus. (N-P) Effects of siRNA on the activation of ERK. \( Fgf8b \)-siRNA decreased the activation level of ERK (N), although \( Fgf8a \)-siRNA hardly affected ERK activity (O). (Q-S) Effects of \( Fgf8b \)-siRNA on \( Otx2 \) expression. The arrows represent the caudal border of the \( Otx2 \) expression domain. Application of \( Fgf8b \)-siRNA by electroporation resulted in a caudal shift of the \( Otx2 \) expression domain; in other words, the mesencephalon extended caudally. (T-V) Effects of \( Fgf8a \)-siRNA on \( Otx2 \) expression. \( Fgf8a \)-siRNA did not affect \( Otx2 \) expression. Dorsal view (B,F,I,N-Q,T), Lateral view (C,D,G,H,K,L,R,U), GFP fluorescence to indicate the site of siRNA introduction (E,I,M,S,V), mes, mesencephalon; met, metencephalon; cont, control side; exp, experimental side. Scale bars: 200 \( \mu \)m.
Fig. 8. Schematic drawing to show the organizing activity of Fgf8 and its signal transduction. Fgf8 is induced at the interface of Otx2 and Gbx2 expression, overlapping with Gbx2 expression. The site where Fgf8 mRNA is localized may receive a strong Fgf8 signal and cause the Ras-ERK pathway to be activated. Thus, this region may acquire the characteristics of rhombomere1 (r1), where cerebellum differentiates. By contrast, in the mesencephalon, the Fgf8-Ras-ERK pathway may be activated only weakly, which may play a role in rostrocaudal polarity formation of the tectum.

because Otx2 is expressed there. Fgf17 and Fgf18 that are induced by Fgf8 together with Fgf8a may regulate proliferation of the mesencephalon and metencephalon (Xu et al., 2000; Liu et al., 2003). In the mesencephalon, ERK activity remains in a gradient distribution after the 10-somite stage, and may cause the Ras-ERK pathway to be activated. Thus, this region may acquire the characteristics of rhombomere1 (r1), where cerebellum differentiates. By contrast, in the mesencephalon, the Fgf8-Ras-ERK pathway may be activated only weakly, which may play a role in rostrocaudal polarity formation of the tectum.

We thank Drs K. Kitamura and M. Wassef for the Otx2 and Wnt1 probes, respectively, Drs K. Moriyoshi and J. Miyazaki for pCA-GAP-GFP, Drs H. Takeda and M. Shinya for helpful suggestions for examining the effect of anti-di-phosphorylated ERK, and Drs Y. Wakamatsu, I. Araki, S. Sugiyama and E. Matsunaga and members of our laboratory for discussions and critical reading of the manuscript. This work was supported by the grants from the Ministry of Education, Culture, Sports, Science and Technology and from the Mitsubishi Foundation. T.S. is a recipient of JSPS Research Fellowships for Young Scientists.

References


