

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

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Accepted 28 May 2004

Development 131, 4275–4285

Published by The Company of Biologists 2004

doi:10.1242/dev.01281

## 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 (*Ras*<sup>S17N</sup>) changed the fate of the metencephalic alar plate from cerebellum to tectum. *Ras*<sup>S17N</sup> canceled the effects of *Fgf8b*, while co-transfection of *Fgf8a* and *Ras*<sup>S17N</sup> 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 isthmus 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 isthmus-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-

mesencephalic region (Lee et al., 1997). By contrast, the mesencephalon of *Fgf8b*-transgenic mice under the control of a *Wnt1* enhancer, showed metencephalic properties (Liu et al., 1999), and *Fgf8b*-soaked beads can induce the development of cerebellar structures in the diencephalon and mesencephalon (Martinez et al., 1999). Moreover, *Fgf8b* misexpressed by in-ovo electroporation completely transformed the fate of the mesencephalic alar plate to become cerebellum. Although *Fgf8a* and *Fgf8b* exerted completely different effects, lower doses of *Fgf8b* exerted similar effects to those of *Fgf8a* (Sato et al., 2001; Liu et al., 2003). This result, together with the result that *Fgf8b* has stronger transforming activity than *Fgf8a* for NIH3T3 cells in vitro (MacArthur et al., 1995a) indicates that the differing effects of *Fgf8* may be attributable to differences in the intensity of the signals.

It is thus of great interest to learn how the Fgf8 signal is transduced in the metencephalon and mesencephalon for their fate decision. In many systems, the signal through a receptor tyrosine kinase (RTK) is transduced through the Ras-extracellular-signal-regulated kinase (ERK) pathway to induce cellular responses, such as proliferation and differentiation (reviewed by Katz and McCormick, 1997; Rommel and Hafen, 1998). Since the Fgf receptor (Fgfr) is also a tyrosine kinase, we focused our attention on the Fgf-Ras-ERK signaling pathway. We first examined ERK activation in chick neural

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 (Ras<sup>S17N</sup>) was carried out to disrupt the Ras-ERK pathway. Ras<sup>S17N</sup> 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'-CATGGATCCATGTACCCATACGACGTCCCAGACTACGCAATG-ACGGAATATAAGCTGG-3' and 5'-AATTAACCCTCACTAAAGGG-3' (T7 primer). PCR products were subcloned into the pBluescript vector and sequenced. *Fgf8a*, *Fgf8b* and HA-Ras<sup>S17N</sup> 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., 1997).

### 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 (CUY21, 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. In some cases, the cathode (Unique Medical Imada) was inserted into the lumen of the neural tube and a rectangular pulse of 15 V, 25 ms was given three times to transfect efficiently in the ventral side of the neural tube.

### Bead 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.

### siRNA to specifically silence *Fgf8a* and *Fgf8b*

Recently, it was shown that siRNA could specifically disrupt target mRNA by introducing siRNA expression vector (Katahira 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 (TTCAAGAGA) 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 *ApaI* restriction site was added to the 5' and 3' end of the reverse oligonucleotides, respectively. The forward and reverse oligonucleotides were annealed, and were 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.

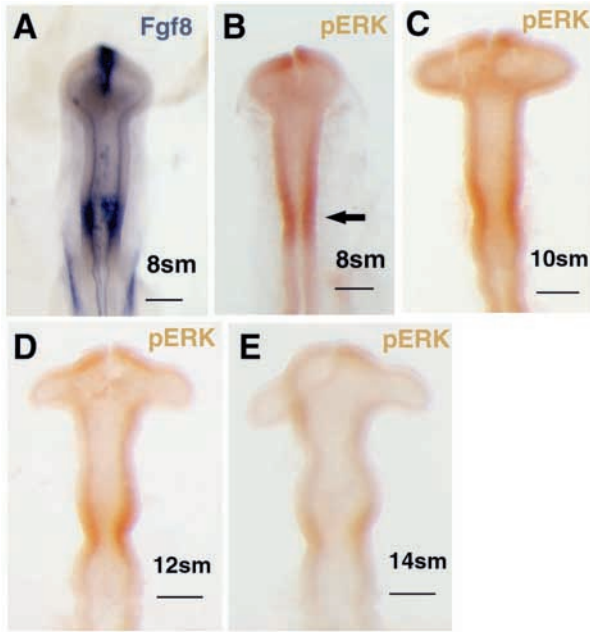
### Histology

Embryos were fixed in 4% paraformaldehyde and embedded in Histoiresin (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 unique TEY sequence (di-phosphorylated ERK, dpERK), and can be detected by an anti-dpERK antibody (Gabay et al., 1997; Christen and Slack, 1999; Shinya et al., 2001; Corson et al., 2003). In the neural tube of embryos at the 8-somite stage (stage 9+, HH9+) (Hamburger and Hamilton, 1951), ERK was activated strongly in the regions where *Fgf8* mRNA was expressed: the isthmus and the anterior neural ridge (Fig. 1A,B). In the MHB region, ERK was activated strongly at the isthmus and weakened rostrally, thus resulting in a gradient of ERK activation (Fig. 1A,B). At the 10-somite stage (HH10, Fig. 1C) and 12-somite stage (HH11–, Fig. 1D), ERK retained activity in the mesencephalon, while its activation became relatively weaker in the metencephalon (Fig. 1C,D). At the 14-somite stage (HH11+), ERK was not activated in the metencephalon but was still activated in the caudal mesencephalon in a gradient: caudal high and rostral low (Fig. 1E).



**Fig. 1.** ERK activation in the neural tube. (A) Whole-mount in-situ hybridization for *Fgf8* at the 8-somite stage (HH9+). (B-E) Distribution of activated ERK revealed immunohistochemically with anti-dpERK antibody in normal embryos. (B) Eight-somite stage (HH9+). (C) Ten-somite stage (HH10). (D) Twelve-somite stage (HH11-). (E) Fourteen-somite stage (HH11+). ERK is activated around the isthmus (arrow on B) and the anterior tip of the neural tube at the 8-somite stage. By the 14-somite stage, ERK has been inactivated in the metencephalon.

### ERK activation by FGF8 signaling

Implantation of an SU5402 bead in the isthmus region was carried out to examine whether ERK activation is dependent on Fgf signaling. SU5402 specifically inhibits the kinase activity of Fgf receptors, but not that of other RTKs (Mohammadi et al., 1997). Observation at 1 or 2 hours after implantation of an SU5402-soaked bead revealed that ERK activation was prevented ( $n=11/13$ ) (Fig. 2A). Thus, we concluded that ERK activation in the MHB is dependent on Fgf signaling.

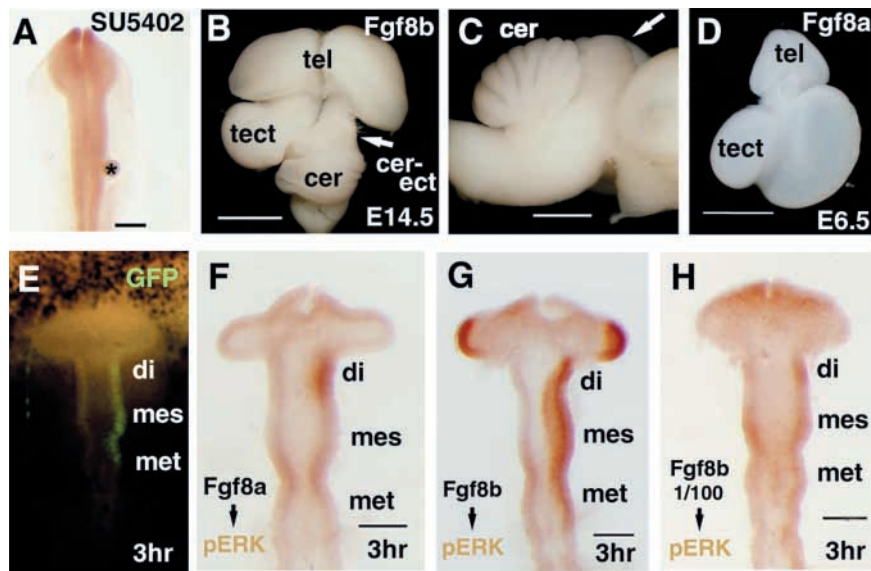
We then proceeded to test whether Fgf signaling could truly activate ERK. For this purpose, misexpression of *Fgf8a* and *Fgf8b* on the right side of the brain vesicles was carried out. As reported previously (Sato et al., 2001), *Fgf8b* instructs the mesencephalic alar plate to differentiate into the 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  $\mu\text{g}/\mu\text{l}$ ) 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 (Ras<sup>S17N</sup>), which was shown to disrupt the Ras-ERK pathway (Feig and Cooper, 1988). Indeed, the activation level of ERK was decreased after misexpression of Ras<sup>S17N</sup> by in-ovo electroporation in 7-10-somite stage embryos ( $n=8/11$ ) (Fig. 3A). Misexpression of Ras<sup>S17N</sup> 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. 3G), 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 Ras<sup>S17N</sup>-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 Ras<sup>S17N</sup>-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 Ras<sup>S17N</sup> was not enough to



**Fig. 2.** ERK activation by Fgf8 signal. (A) Immunohistochemistry with anti-dpERK 1 hour after insertion of a 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  $\mu\text{g}/\mu\text{l}$  (F), *Fgf8b* 1  $\mu\text{g}/\mu\text{l}$  (G), *Fgf8b* 0.01  $\mu\text{g}/\mu\text{l}$  (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  $\mu\text{g}/\mu\text{l}$  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  $\mu\text{m}$  (A-F-H), 4 mm (B-D).

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*<sup>S17N</sup>. 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 *Ras*<sup>S17N</sup> 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 *Ras*<sup>S17N</sup> (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 *Ras*<sup>S17N</sup>, 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 *Ras*<sup>S17N</sup>-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).

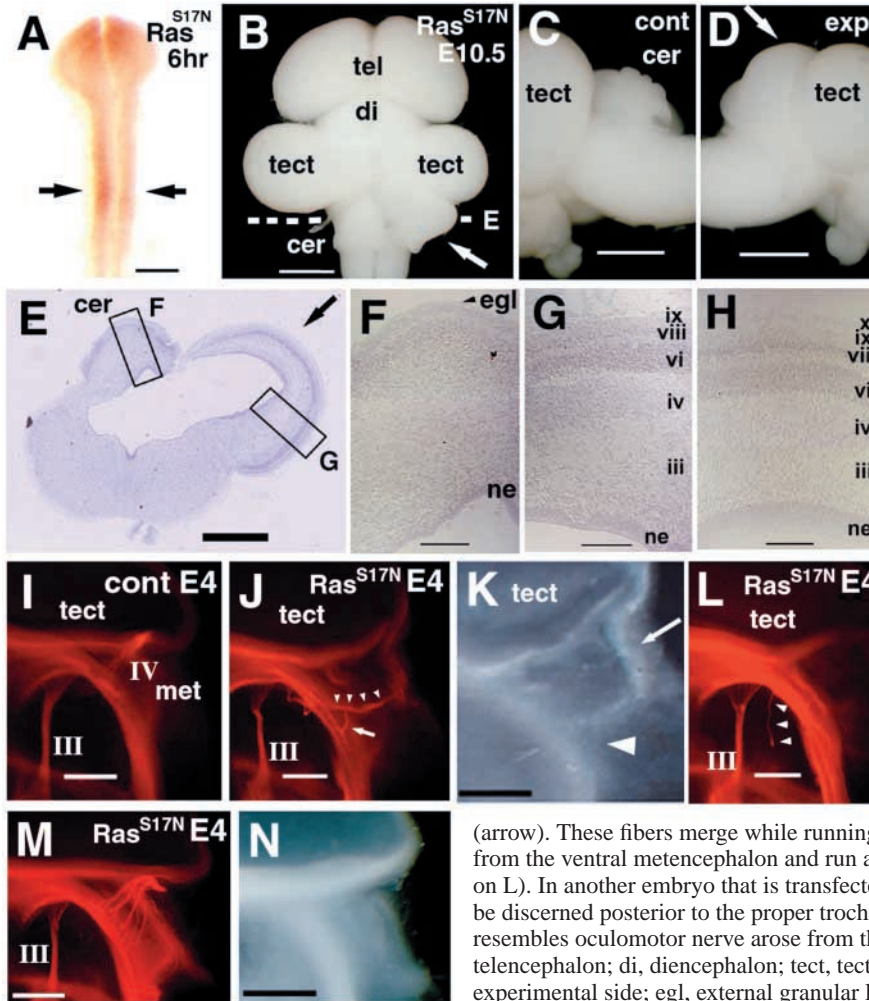
Next, we examined the effect on *Wnt1* expression. At E2.5, *Wnt1* was expressed in the dorsal midline of the mesencephalon and in the caudal metencephalon. Since the dorsal midline of the metencephalon does not express *Wnt1*, it is a good marker to discriminate between metencephalon and mesencephalon (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 *Ras*<sup>S17N</sup> 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 *Ras*<sup>S17N</sup> 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 *Ras*<sup>S17N</sup> 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 *Ras*<sup>S17N</sup>. If Ras functions at the downstream of the Fgf8b signal, co-transfection of *Ras*<sup>S17N</sup> with *Fgf8b* may cancel the effects of



**Fig. 3.** Fate change of the metencephalon to the mesencephalon by Ras<sup>S17N</sup> misexpression. (A) Repression of ERK activity by misexpression of Ras<sup>S17N</sup>. (B-D) Views from the dorsal (B), left (C) and right (D) of the E10.5 chick brain after misexpression of Ras<sup>S17N</sup>. The tectum differentiated ectopically in place of the cerebellum (arrow on B,D). Posterior part differentiated into the cerebellum according to the original program (arrowheads on D). (E) A transverse section of the brain at the level of (A). (F,G) Higher magnification of the cerebellum and ectopic tectum indicated as (F,G, respectively), on (D). The external granular layer is differentiating on the control side (arrowhead on F). (H) Higher magnification of the proper tectum. The ectopic tectum has nine layers (G), while the proper tectum has ten layers (H). (I,J,L,M) Whole-mount immunohistochemistry with anti-neurofilament antibody on E4 embryos. (K,N) Bright field of (J,M, respectively). An arrow and arrowhead on (K) indicate a large ectopic swelling and a small one in the metencephalon, respectively. In normal embryos (I), oculomotor (III) and trochlear (IV) nerves are discernible. In Ras<sup>S17N</sup>-transfected embryos (J), trochlear nerve fibers arise from the posterior part of the large swelling and run dorsally (arrowheads). Additional nerve fibers arise from the posterior part of the small swelling (arrow). These fibers merge while running dorsally. In some cases, a nerve trunk originates from the ventral metencephalon and run a similar course to the oculomotor nerve (arrowheads on L). In another embryo that is transfected with Ras<sup>S17N</sup>, several additional nerve fibers could be discerned posterior to the proper trochlear nerve. In some embryos, the nerve trunk that resembles oculomotor nerve arose from the metencephalic region (M, arrowheads). tel, telencephalon; di, diencephalon; tect, tectum; cer, cerebellum; cont, control side; exp, experimental side; egl, external granular layer; ne, neuroepithelium; III, oculomotor nerve; IV, trochlear nerve. Scale bars: 4 mm (B,C,D); 2 mm (E); 200  $\mu$ m (A,F,G,H); 400  $\mu$ m (I-N).

*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 ( $n=4/4$ ) (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 expression of the introduced gene product, explaining why cerebellar differentiation may have occurred in this region.

Co-transfection of *Fgf8a* and Ras<sup>S17N</sup> exerted additive effects ( $n=4/4$ ), whereby there was differentiation of the ectopic tectum in the diencephalon (Fig. 6G,H) and in the metencephalon (Fig. 6I). These data suggest that activation of Ras signaling is necessary for cerebellar development, but is not crucial for tectal differentiation.

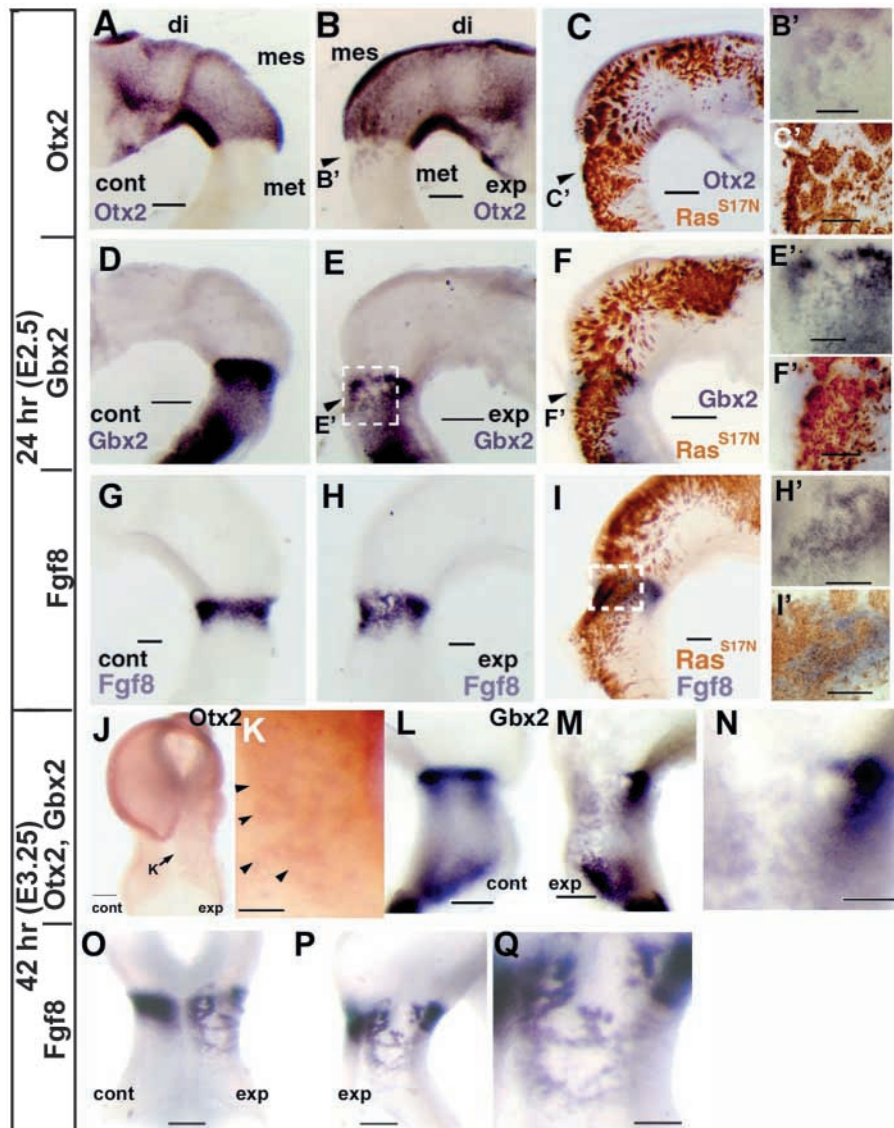
#### Differential silencing of *Fgf8a* and *Fgf8b* by siRNA method

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 ( $n=5/7$ ) (Fig. 7B-E). Degradation of *Fgf8* mRNA by Fgf8a-siRNA could not be detected ( $n=8/8$ ) (Fig. 7F-I). Efficient degradation of *Fgf8* mRNA could be observed after application of a mixture of Fgf8a- and Fgf8b-siRNA ( $n=3/5$ ) (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 ( $n=7/10$ ) (Fig. 7N) and of both Fgf8a- and Fgf8b-siRNA ( $n=7/11$ ) (Fig. 7P). Fgf8a-siRNA alone did not affect



**Fig. 4.** Effects of Ras<sup>S17N</sup> misexpression on *Otx2*, *Gbx2* and *Fgf8*. Effects on *Otx2* (A-C,J,K), *Gbx2* (D-F,L-N) and *Fgf8* (G-I,O-Q). Misexpression site of Ras<sup>S17N</sup> (brown in C,F,I) is assessed by immunohistochemistry against HA-tag. *Otx2*, *Gbx2* and *Fgf8* expression is represented as blue by in-situ hybridization. Twenty-four and 42 hours after electroporation (A-I and J-Q, respectively). Left (control) side of the brain vesicles (A,D,G,L) and right (transfected) side (B,C,E,F,H,I,M,P). Dorsal view (J,O). Higher magnification (B',C',E',F',H',I',K,N,Q). The rectangular area on (E) corresponds to (E'). By 24 hours after electroporation, *Otx2* was induced in the metencephalon by Ras<sup>S17N</sup> misexpression (B,C,B',C'). *Gbx2* was repressed in the metencephalon by Ras<sup>S17N</sup> misexpression (E,F,E',F'). Endogenous *Fgf8* expression was also repressed, but was induced in the periphery of Ras<sup>S17N</sup> expression in the metencephalon (H,I,H',I'). By 42 hours after electroporation, the boundary of patchy expression of *Otx2* in the metencephalic region became blurred (J,K), and *Otx2* expression area expanded (arrowheads in K). Repression of *Gbx2* and *Fgf8* just posterior to the proper mesencephalon became wider, leaving a *Gbx2*- and *Fgf8*-free region (L-Q). di, diencephalon; mes, mesencephalon; met, metencephalon; cont, control side; exp, experimental side. Scale bars: 200  $\mu$ m (A-C,D-F,G-I,J,L,M,O,P), 100  $\mu$ m (B',C',E',F',H',I',K,N,Q).

the activation level of ERK ( $n=11/14$ ) (Fig. 7O). Ras<sup>S17N</sup> 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 Ras<sup>S17N</sup> 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, Ras<sup>S17N</sup> 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 region ( $n=4/14$ ) (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

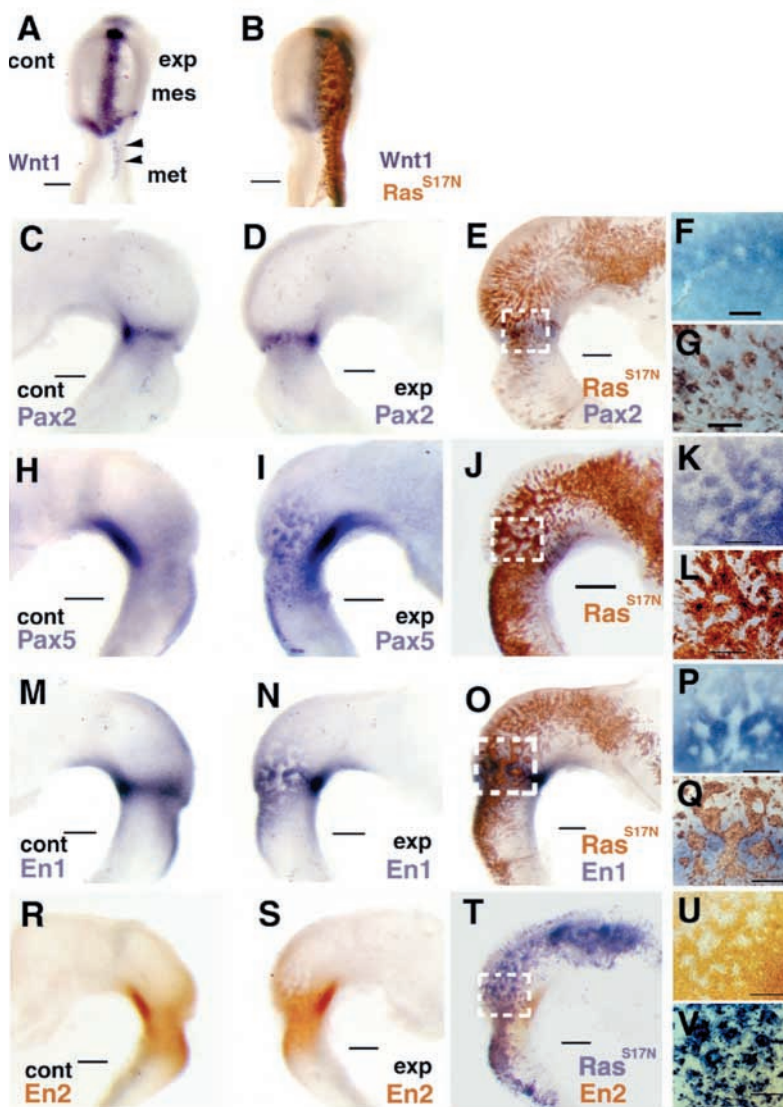
to the bead with tectum around the mini cerebellum (Martinez et al., 1999). Transgenic mice in which *Fgf8b* was misexpressed under the control of a *Wnt1* enhancer changed the property of the presumptive diencephalon and mesencephalon to that of the metencephalon (Liu et al., 1999). Moreover, misexpression 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  $\mu\text{g}/\mu\text{l}$  resulted in activation of ERK at *Fgf8b* misexpressing sites throughout the diencephalon

and metencephalon, while misexpression of *Fgf8b* at 0.01  $\mu\text{g}/\mu\text{l}$  or *Fgf8a* at 1  $\mu\text{g}/\mu\text{l}$  resulted in activation of ERK in only a portion of the diencephalon.

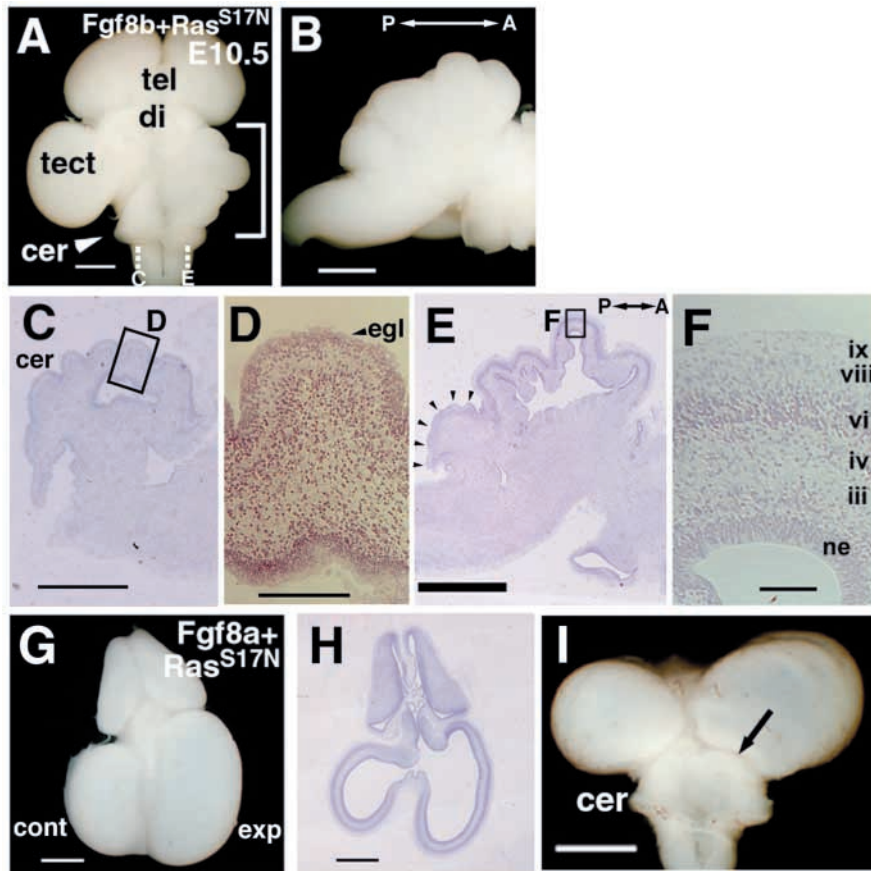
Ras, a member of a group of small GTP-binding proteins, is activated downstream of various RTKs and activates Raf, Mek and ERK in turn (reviewed by Katz and McCormick, 1997; Rommel and Hafen, 1998). Since ERK was activated in the isthmus and sites of *Fgf8b* misexpression, we assumed that activation 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 (*Ras*<sup>S17N</sup>) 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 *Ras*<sup>S17N</sup>-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 *Ras*<sup>S17N</sup>, 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



**Fig. 5.** Effects of *Ras*<sup>S17N</sup> 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 *Ras*<sup>S17N</sup> misexpression. Scale bars: 200  $\mu\text{m}$ .



**Fig. 6.** Co-transfection of *Fgf8b/a* with *Ras<sup>S17N</sup>*. (A–F) Dorsal view (A) and lateral view (B), parasagittal section at the line indicated on (A) as (C) and (E) (C,E) of E10.5 brain co-electroporated with *Fgf8b* and *Ras<sup>S17N</sup>* at the 10-somite stage. (D,F) higher magnification of (C,E, respectively). The swelling in the mesencephalic region and anterior metencephalic region of the experimental side have sulci on the surface, and look like cerebellum (A,B). Histologically, however, the swelling in the mesencephalon and the anterior metencephalon of the experimental side do not have an external granular layer, which is seen in the cerebellum of the control side (C,D), and has tectal architecture (E,F). The posterior part of the metencephalic region consists of cerebellar structure (arrowheads on E). Antero-posterior direction is indicated by the double-headed arrow on (B,E). Dorsal view (G,I) and horizontal section (H) of the brain co-electroporated with *Fgf8a* and *Ras<sup>S17N</sup>*. The tectum has extended to the diencephalic territory (G,H). Tectum differentiated ectopically in the metencephalon (I, arrow). tel, telencephalon; di, diencephalon; tect, tectum; cer, cerebellum; egl, external granular layer; tect, tectum; ne, neuroepithelial layer; roman numerals on (F) indicate the tectal layers. Scale bars: 4 mm (A,B,C,E,G,H,I); 200  $\mu$ m (D,F).

the Ras-ERK pathway is activated by *Fgf8b* to result in metencephalic differentiation comes from co-transfection studies with *Ras<sup>S17N</sup>* 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 *Ras<sup>S17N</sup>* and *Fgf8b* canceled the effects of *Fgf8b* misexpression, while co-transfection of *Fgf8a* and *Ras<sup>S17N</sup>* caused differentiation of the ectopic tectum in the diencephalon and in the metencephalon, displaying the additive effects of *Fgf8a* and *Ras<sup>S17N</sup>* 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 (Meyers et al., 1998; Reifers et al., 1998; Chi et al., 2003), it was thought that *Fgf8* might also be necessary for the development of the mesencephalon. However, our results show that disruption of the Ras signaling pathway did not affect the fate of the presumptive mesencephalon, and actually changed the fate of the presumptive metencephalon to that of the mesencephalon. This suggests that Ras signaling does not play a role in fate

determination of the mesencephalon. To accord our assumption, animal cap assay indicated that the PLC $\gamma$  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.

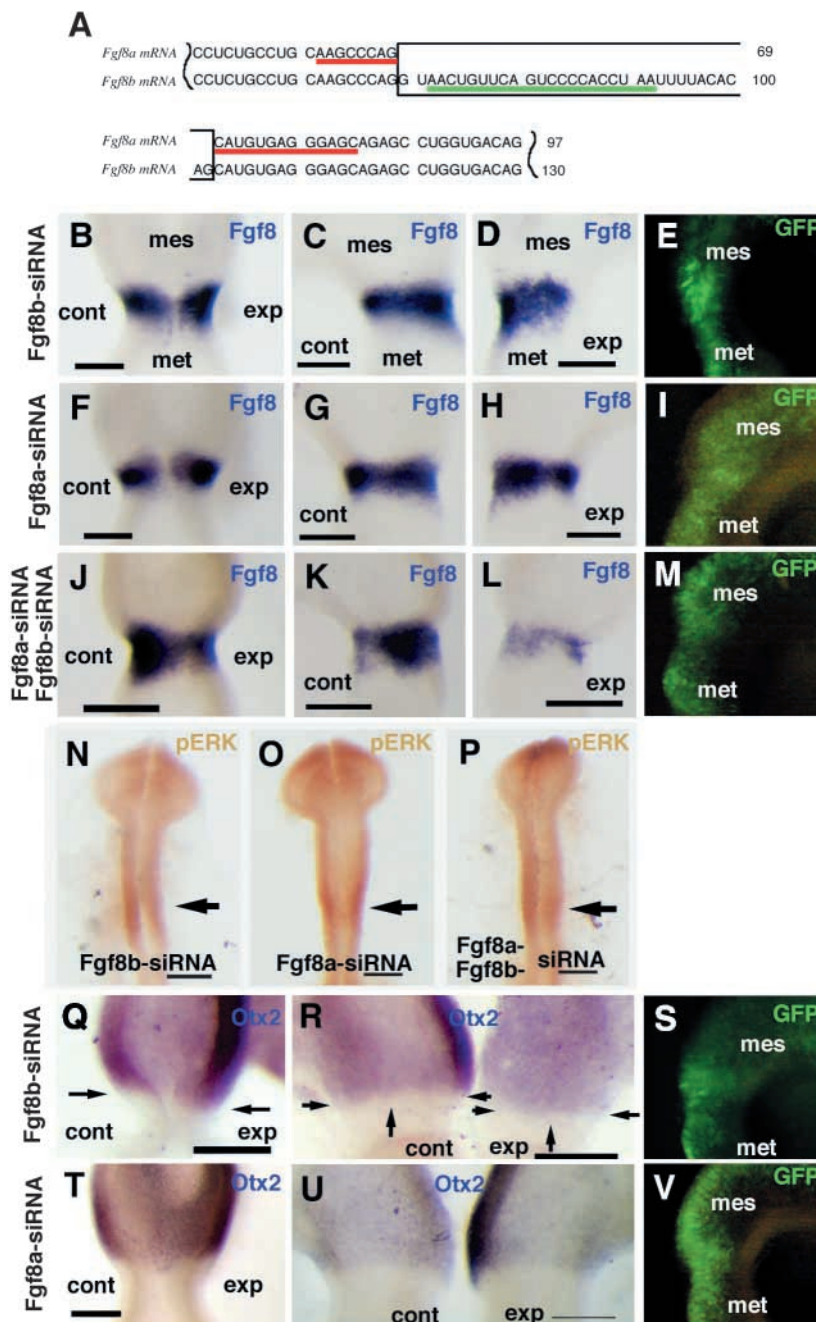
In the mesencephalon, the Fgf8-Ras-ERK signaling pathway may be involved in rostrocaudal polarity formation. For the rostrocaudal polarity formation, it is suggested that En confers caudal property to the tectal anlage so that the rostrocaudal polarity of the tectum is determined according to a gradient 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

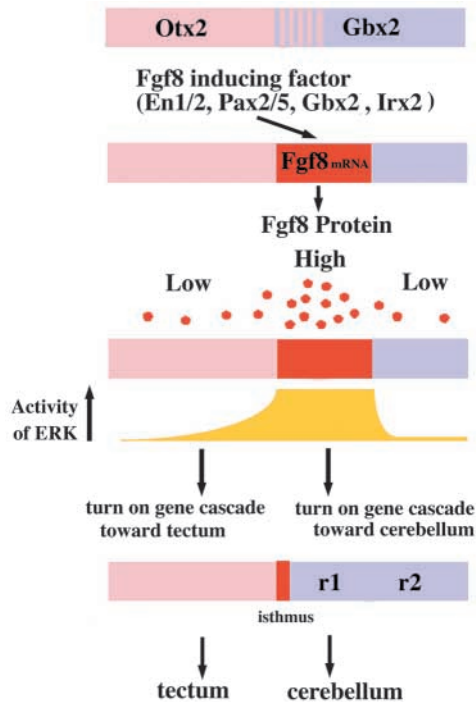
indicates that the gene expression cascade favoring cerebellar differentiation has proceeded by the 10-somite stage, meaning that the fate of the metencephalon is determined by this time. This notion is supported by ectopic transplantation studies that show that while the rostrocaudal axis of the mesencephalon 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; Irving and Mason, 2000; Katahira et al., 2000; 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,J,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 contribute to the determination of the rostrocaudal axis 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.

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