# Regulation of isthmic Fgf8 signal by *sprouty2*

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## Summary

**Fgf8 functions as an organizer at the mes/metencephalic boundary (isthmus). We showed that a strong Fgf8 signal activates the Ras-ERK signaling pathway to organize cerebellar differentiation. Sprouty2 is expressed in an overlapping manner to Fgf8, and is induced by Fgf8. Its function, however, is indicated to antagonize Ras-ERK signaling. Here, we show the regulation of Fgf8 signaling in relation to Sprouty2. *sprouty2* expression was induced very rapidly by Fgf8b, but interfered with ERK activation. *sprouty2* misexpression resulted in a fate change of the presumptive metencephalon to the mesencephalon. Misexpression of a dominant negative form of Sprouty2 augmented ERK activation, and resulted in anterior shift of the posterior border of the tectum. The results indicate that Fgf8 activates the Ras-ERK signaling pathway to differentiate the cerebellum, and that the hyper- or hypo-signaling of this pathway affects the fate of the brain vesicles. Sprouty2 may regulate the Fgf8-Ras-ERK signaling pathway for the proper regionalization of the metencephalon and mesencephalon.**

Key words: Fgf8, Chick, Sprouty, Isthmus

## Introduction

*Fgf8* is expressed in the isthmus and acts as an organizer for the mesencephalon and metencephalon (Crossley and Martin, 1995; Crossley et al., 1996; Martinez et al., 1999; Nakamura, 2001; Sato et al., 2001). Sato et al. (Sato et al., 2001) showed that *Fgf8a* and *Fgf8b* are expressed in the isthmus, and that *Fgf8b* could change the fate of the presumptive mesencephalon to that of the metencephalon; that is, the cerebellum differentiated in place of the tectum after *Fgf8b* misexpression by electroporation. Very recent work showed that the Fgf8b signal is transduced by the Ras-ERK signaling pathway to organize cerebellar differentiation. If the Ras-ERK signaling pathway is disrupted by misexpression of a dominant negative form of Ras, the presumptive metencephalon changes its property to that of the mesencephalon (Sato and Nakamura, 2004).

*sprouty2* is expressed in the isthmus (Chambers and Mason, 2000; Chambers et al., 2000; Zhang et al., 2001; Lin et al., 2002; Liu et al., 2003). Although *sprouty* is expressed overlapping *Fgf8*, and could be induced by an Fgf signal, it is suggested that *sprouty2* functions as a negative regulator of the Fgf-Ras-ERK signaling pathway (Hacohen et al., 1998; Casci et al., 1999; Kramer et al., 1999; Lin et al., 2002). The negative feedback of the Fgf8 signaling pathway by Sprouty2 is controversial, but is very interesting from the view of Fgf8 signal transduction and regulation.

We carried out misexpression of *Fgf8b*, *sprouty2* and a dominant negative form of *sprouty2* (*sprouty2-DN*). Our results indicate that *sprouty2* expression is induced very rapidly by Fgf8b, and that Sprouty2 interferes with ERK activation. We have also shown that *sprouty2* misexpression resulted in the fate change of the presumptive metencephalon to the mesencephalon. Misexpression of dominant negative form of Sprouty2 resulted in an anterior shift of the posterior border of the tectum. The results indicate that Fgf8 activates the Ras-ERK signaling pathway to differentiate the cerebellum, and that the hyper- or hypo-signaling of this pathway affects the fate of the brain vesicles. Thus, Sprouty2 may regulate the Fgf8-Ras-ERK signaling pathway for the proper regionalization of the metencephalon and mesencephalon.

## Materials and methods

### Expression vector and in ovo electroporation

The full length *sprouty2* cDNA was isolated from E2 chick by RT-PCR, and inserted in pBluescript (pBluescript-Sprouty2). Primers for N- and C-terminal fragments are 5′-GATGTGTTCTAAGCCTGC-TGG-3′ and 5′-AGTGCCAAGACCATAGCTGC-3′, respectively. *sprouty2*-DN was created by substituting alanine for tyrosine 55 of *sprouty2* (Sasaki et al., 2001). For this, QuikChange Site-Directed Mutagenesis Kit (STRATAGENE) was used. PfuTurbo DNA polymerase$^{II}$ in the kit replicates both forward and reverse strands of pBluescript-Sprouty2 with primers that contain mutation (5′-GC-AACACGAATGAGGCCACAGAGGGACCGACG-3′ and 5′-CGTC-GGTCCCTCTGTGGCCTCATTCGTGTT-3′, underlining indicates mutation). *sprouty2* and HA-tagged *sprouty2*-DN (Sprouty2-DN) were inserted into pMiwIII, which has Rous sarcoma virus enhancer and chicken β-actin promoter (Suemori et al., 1990; Wakamatsu et al., 1997; Mastunaga et al., 2001).

*Fgf8a* and *Fgf8b* expression vectors were prepared by Sato (Sato

et al., 2001). For transfection, in ovo electroporation was carried out at HH 8-9 (stage 8-9) (for details, see Hamburger and Hamilton, 1951) as described previously (Funahashi et al., 1999; Nakamura and Funahashi, 2001). GFP expression vector was co-electroporated to check the efficiency of transfection.

### Morpholino antisense oligonucleotide

Fluorescein-labeled morpholino antisense oligonucleotide against Sprouty2 was applied by electroporation as described previously (Sheng et al., 2003; Sugiyama and Nakamura, 2003).

### Histology

Embryos were fixed in 4% paraformaldehyde in PBS (phosphate buffered saline), and embedded in Technovit (Kulter). Serial 5 μm sections were stained with Hematoxylin and Eosin.

### In situ hybridization and immunohistochemistry

In situ hybridization was carried out according to Wilkinson (Wilkinson, 1992). Probes for *Otx2, Gbx2, Fgf8, Wnt1, Lmx1b* and mouse *Fgf8* are described in Katahira et al. (Katahira et al., 1999), Matsunaga et al. (Matsunaga et al., 2002) and Funahashi et al. (Funahashi et al., 1999). The template for *sprouty2* probe, consisting of 129 bp of 5′UTR and 467 bp of 5′ coding region, was subcloned into pBluescript. Digoxigenine (DIG)- or fluorescein isothiocyanate (FITC)-labeled RNA probes were transcribed by T3 or T7 RNA polymerase according to the manufacturer's protocol. Alkaline phosphatase (ALP)-conjugated anti-FITC or anti-DIG antibodies were used for detection, and were colored by Fast Red/Naphtol AS/MX, and nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), respectively. For some cases, Fast Red/Naphtol AS/MX was washed away in ethanol.

For immunohistochemistry, anti-HA rabbit polyclonal antibody (Berkeley Antibody Company), anti-neurofilament monoclonal antibody, 3A10 (DSHB), and anti-diphosphorylated ERK antibody (Sigma) were used as primary antibodies. As secondary antibodies, horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Jackson), Cy3-conjugated anti-mouse IgG (Jackson), and biotinylated anti-mouse IgG antibody (VECTOR) were used. HRP was detected with 3,3′-diaminobenzidine (DAB). For detection of biotinylated antibody, the ABC-Elite system (VECTOR) was adopted.

## Results

### Normal expression pattern of Fgf8 and Sprouty2

First, we examined the normal expression pattern of *Fgf8* and *sprouty2*. As reported previously, *Fgf8* was expressed in the rostral tip of the forebrain and the isthmus at HH 11. *sprouty2* was expressed in an overlapping manner to *Fgf8* throughout the stages examined (Fig. 1). The expression pattern indicates an intimate relationship between *Fgf8* and *sprouty2*. Indeed, it has been reported that the Fgf8 bead could induce *sprouty2* expression (Minowada et al., 1999; Chambers et al., 2000).

### Induction of Sprouty2 by Fgf8

It has been reported that *sprouty2* is induced by Fgf8, but that Sprouty2 functions as a negative regulator of Fgf signaling (Hacohen et al., 1998; Kramer et al., 1999; Casci et al., 1999; Lin et al., 2002). We wanted to see if *sprouty2* is really induced by *Fgf8,* and functions as a negative regulator of the Ras-ERK signaling pathway. It was surprising that *sprouty2* was already induced by 3 hours after electroporation of both *Fgf8a* and *Fgf8b* expression vector (HH 10). At 3 hours after electroporation of *Fgf8a* and *Fgf8b* expression vector (HH 9), *Fgf8* misexpression could be seen in large areas of the mesencephalon, metencephalon and myelencephalon (Fig. 2A,C,I,K). *sprouty2* was already induced in an overlapping manner to *Fgf8a* and *Fgf8b* expression (Fig. 2B,D,J,L; *Fgf8a*; *n*=2/3, *Fgf8b*; *n*=7/9). Induction of *sprouty2* by *Fgf8* is very rapid as suggested by Chambers et al. (Chambers et al., 2000), and occurs around 1 hour if we consider that translation product is expressed by 2 hours after electroporation (Funahashi et al., 1999).

At 24 hours after electroporation with *Fgf8a* expression vector at a concentration of 1 μg/μl (HH 18), *Fgf8* misexpression was well discerned. *sprouty2* expression was induced in Fgf8 misexpression sites at the lateral side of the mesencephalon (Fig. 2E-H; *n*=7/8).

At 24 hours after electroporation with *Fgf8b* expression vector at a concentration of 1 μg/μl (HH 18), misexpression of *Fgf8b* was widely discernible from the metencephalon to the diencephalon. In addition to the misexpression at the lateral side of the neural tube, as was seen after *Fgf8a* misexpression, strong line-like expression of *Fgf8* was discernible along the roof plate of the mesencephalon (Fig. 2M, *n*=15/16). *sprouty2* was induced in a similar pattern to *Fgf8* expression (Fig. 2N), but in a little wider region than that of *Fgf8* at the lateral side of the neural tube (Fig. 2O,P). Induction of *sprouty2* along the roof plate was also visible (Fig. 2N).

At 24 hours after electroporation with 0.1 μg/μl of expression vector (HH 18), which also changes the fate of the mesencephalon to the metencephalon, strong V-shaped expression of *Fgf8* and *sprouty2* was discernible (Fig. 2Q,R, *n*=3/5). Differential hybridization revealed that introduced *Fgf8* was hardly detected, but the V-shaped *Fgf8* expression was the transcripts from the embryonic gene (*n*=2/2).

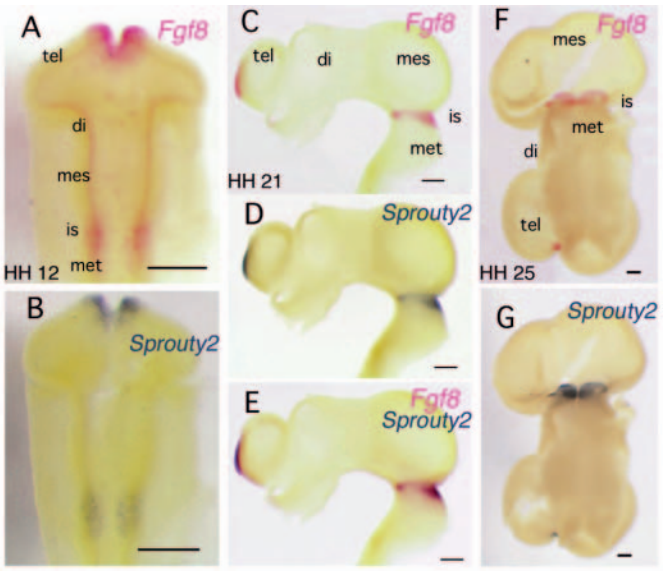


**Fig. 1.** *sprouty2* and *Fgf8* expression in normal embryos. (A,C,F) In situ hybridization for *Fgf8*; (B,D,G) in situ hybridization for *sprouty2*; (E) in situ hybridization for both *Fgf8* and *sprouty2*, at HH 12 (A,B), HH 21 (C-E), HH 25 (F,G). In all the embryos examined, Sprouty2 was expressed overlapping *Fgf8*, the anterior neural ridge and the isthmus. The ages of the embryos are indicated. tel, telencephalon; di, diencephalon; mes, metencephalon; is, isthmus; met, metencephalon, HH represents the stage of Hamburger and Hamilton (Hamburger and Hamilton, 1951), scale bar: 200 μm.

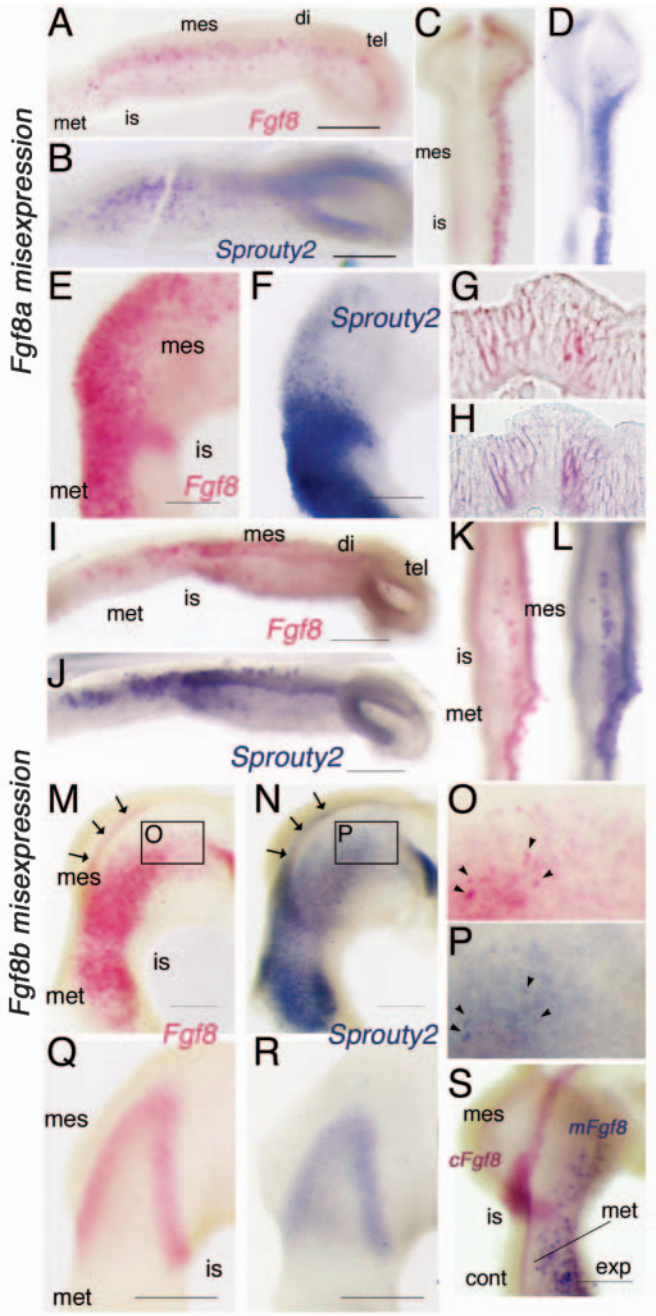


**Fig. 2.** Induction of *Sprout2* by Fgf8. *sprouty2* induction at 3 hours after *Fgf8a* misexpression (A-D, the same embryo), and 24 hours after electroporation (E-H). E and F are form the same embryo, and G and H show the same section at the mesencephalon. (I-L,M-R) *sprouty2* induction at 3 hours after *Fgf8b* misexpression (I-L, the same embryo), and 24 hours after electroporation (M-R, M,N and Q,R are from the same embryo). O and P are higher magnifications of the areas indicated as O and P on M and N, respectively. (S) Electroporation of mouse *Fgf8b* and hybridization with *cFgf8* and *mFgf8* probes that do not cross hybridize each other. In situ hybridization for *Fgf8* (A,C,E,G,I,K,M,O,Q), for *sprouty2* (B,D,F,H,J,L,N,P,R). At 3 hours after electroporation with *Fgf8a* vector (HH 9), misexpression of *Fgf8* is easily discernible (A,C), and *sprouty2* is induced overlapping *Fgf8* expression (B,D). At 24 hours after electroporation, *Fgf8* misexpression can be discerned in a wide area (E). *sprouty2* is expressed overlapping *Fgf8* (E-H). At 3 hours after electroporation with *Fgf8b* expression vector, overlapping expression of *sprouty2* and *Fgf8* is easily discernible (I-L). At 24 hours after electroporation of chick Fgf8 vector (1 μg/μl, M,N) (HH 18), *Fgf8* and *sprouty2* expression is widely discernible from the metencephalon to the diencephalon at the lateral side of the mesencephalon (M,N). Strong line-like expression of *Fgf8* and *sprouty2* along the roof plate of the mesencephalon was also seen (arrows on M and N). Misexpression of mouse *Fgf8* (purple, S) and hybridization with chick *Fgf8* (red, S) show that the expression along the roof plate is of the transcripts from the embryonic gene. Higher magnification figures show that *sprouty2* expression is induced slightly more widely than the *Fgf8* expression area (O,P). The arrowhead on O and P indicate the same point. At 24 hours after electroporation of chick Fgf8 vector at a concentration of 0.1 μg/μl, V-shaped expression of *Fgf8* and *sprouty2* is seen (Q,R). The right-hand-side on the dorsal view represents the experimental side. tel, telencephalon; di, diencephalon; mes, mesencephalon; is, isthmus; met, metencephalon; cont, control side; exp, experimental side. Scale bar: 200 μm.

We supposed that *Fgf8b* misexpression in a condition that changes the presumptive mesencephalon to the metencephalic property resulted in new isthmus formation, and that *Fgf8* and *Sprouty* gene misexpression along the roof plate may represent new isthmus, that is, *Fgf8* mRNA in the new isthmus may have been transcribed from the embryonic gene. So, we tried to distinguish *Fgf8* mRNAs between those transcribed from transfected *Fgf8* and those transcribed from the endogenous one. For this purpose, we electroporated *mouse Fgf8b* expression vector and hybridized the embryos differentially with chick and mouse *Fgf8* probes. Differential hybridization revealed that *Fgf8b* misexpression along the roof plate is the transcript from the embryonic gene (Fig. 2S, *n*=5/6). Introduced mouse *Fgf8* was observed widely on the lateral part of the mesencephalon, but not near the roof plate.

## Reconstruction of the isthmus after Fgf8b misexpression

Since the new *Fgf8* expression line was set after *Fgf8b* misexpression, we carried out time course analysis of the reconstruction of the isthmus. The posterior limit of the *Otx2* expression domain gradually retreated toward the anterior (Fig. 3A; 3, 6 and 9 hours after electroporation, *n*=2/3, 5/5 and 5/7 respectively). On the contrary, the *Gbx2* expression domain extended anteriorly complementary to the *Otx2* expression domain after *Fgf8b* misexpression (Fig. 3B; 3, 6 and 9 hours after electroporation, *n*=2/2, 3/4 and 3/3, respectively).

Isthmic *Fgf8* expression had been kept until 6 hours after electroporation of *Fgf8b* expression vector (Fig. 3C, parts a and b, *n*=4/4), but expression became very weak by 9 hours after electroporation (Fig. 3C, part c, *n*=5/6), and disappeared by 12 hours after electroporation (*n*=3/4). New *Fgf8* expression line, which had been transcribed from the embryonic gene, appeared near the roof plate in the embryos 24 and 36 hours after electroporation of *Fgf8b* expression vector (Fig. 2M-S and Fig. 3C, part f, *n*=12/16). *sprouty2* expression was in a similar pattern to *Fgf8* expression (3, 6 and 9 hours after electroporation, *n*=7/9, 2/2 and 3/3, respectively).

We also examined *Limx1b* expression. Limx1b was shown to repress *Fgf8* in a cell-autonomous fashion, but induces *Fgf8*

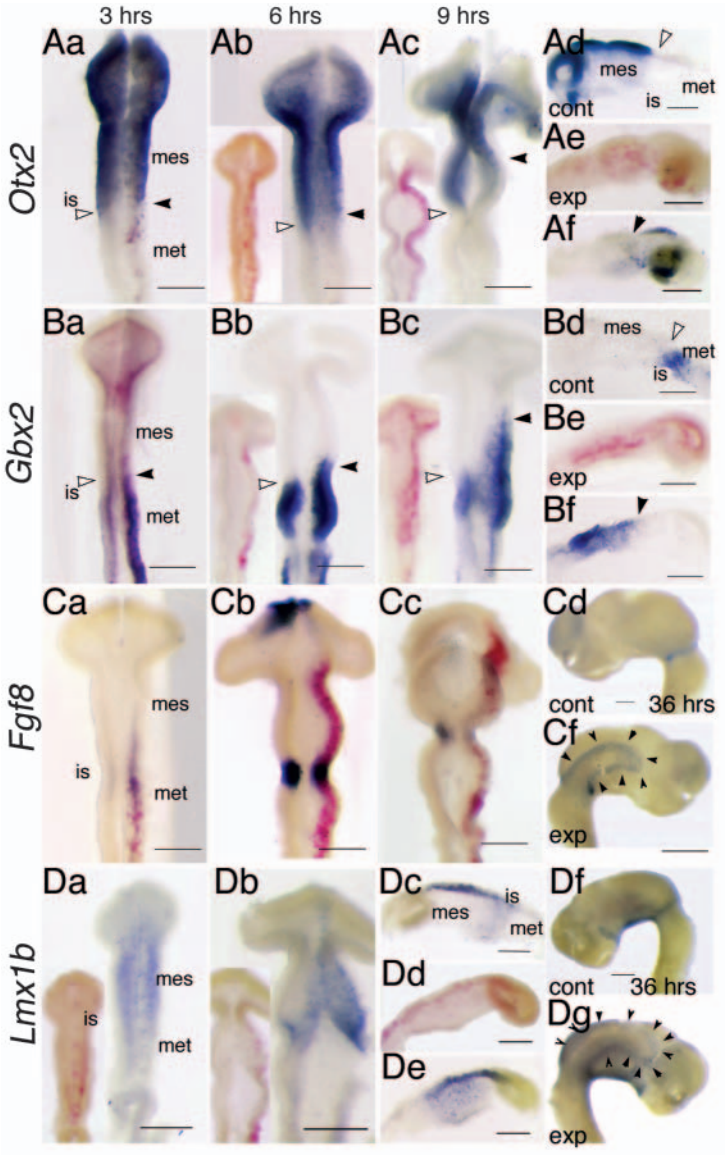


**Fig. 3.** Reconstruction of the isthmus after *mouse Fgf8b* misexpression. (A) In situ hybridization for *Otx2* (purple), in situ hybridization for *mouse Fgf8* (red): A, part e and inlet of A, parts b and c. Arrowhead indicates the posterior limit of the *Otx2* expression domain. Photos of A, parts c-f are from the same embryo. (B) in situ hybridization for *Gbx2* (purple), in situ hybridizaition for *mouse Fgf8* (red): B, part e and inlet of B, parts b and c. Arrowhead indicates anterior limit of *Gbx2* expression domain. Photos of B, parts c-f are from the same embryo. (C) Double in situ hybridization with *chick Fgf8* probe (purple), and with *mouse Fgf8* probe (red). Arrowheads on C, part f indicate *chick Fgf8* (endogenous) expression line. (D) in situ hybridization for *Lmxb1* (purple), in situ hybridization for *mouse Fgf8* (red): D, part d and inlet of D, parts b and c. Arrowheads on D, part g indicate *Limx1b* expression line. *Otx2* expression in the mesencephalon retreats (A), and complementarily, *Gbx2* expression extends rostrally (B). *Fgf8* expression in the isthmus, that is expression from the embryonic gene (purple on C), disappears, and a new expression line appears by 36 hours after electroporation. Interaction of among Fgf8, Lmx1b and Wnt1 may be involved in reconstruction of the isthmus as indicated by Matsunaga et al. (Matsunaga et al., 2003). mes, mesencephalon; is, isthmus; met, metencephalon; exp, experimental side; cont, control side. Scale bar: 200 μm.

expression around *Limx1b*-expressing cells (Matsunaga et al., 2002). *Limx1b* was induced by misexpressed Fgf8b (Fig. 3D, parts a and b, *n*=4/4), but *Limx1b* expression in the mesencephalon became gradually repressed Fig. 3D, parts c-e, *n*=3/3), and ring-like expression of *Limx1b* remained in the diencephalon by 36 hours after electroporation (Fig. 3D, parts f and g, *n*=2/3) (Matsunaga et al., 2002).

## Sprouty2 acts as a negative regulator of the Ras-ERK signaling pathway

It has recently been shown that the Fgf8b signal is transduced by the Ras-ERK signaling pathway to organize the cerebellar differentiation (Sato and Nakamura, 2004). We have shown that *sprouty2* is induced very rapidly by Fgf8, but it is indicated that Sprouty2 acts as a negative regulator (Hacohen et al., 1998; Casci et al., 1999; Kramer et al., 1999; Lin et al., 2002). Then we wondered whether Sprouty2 really acts as a negative regulator for the Ras-ERK signaling pathway, and examined the effects of *sprouty2* on ERK activity. Activated ERK is phosphorylated at both threonine and tyrosine residues that lie adjacent to each other in the unique TEY sequence (diphosphorylated ERK, dpERK), and could be distinguished by the anti-dpERK antibody (Gabay et al., 1997; Christen and Slack, 1999; Shinya et al., 2001).

Time course analysis of the effects of Sprouty2 and the dominant negative form of Sprouty2 on ERK activity was carried out. In the control side, decrease in the ERK activation zone could be recognized during the time course examined (Fig. 4A-C). Repression of ERK activity was already discernible at 3 hours after electroporation of Sprouty2 expression vector (Fig. 4A, *n*=3/5). Repression became stronger as time passed, and by 9 hours after electroporation, very strong repression was discernible as assessed by immunohistochemistry with anti-dpERK antibody (Fig. 4C,D, *n*=6/6).

On the other hand, repression of Sprouty2 activity by antisense morpholino oligonucleotide against Sprouty2 (Fig. 4E, *n*=2/2) or by *sprouty2-DN* misexpression (Fig. 4F-H, *n*=14/15), augmented the activation level of ERK. Although the activation zone of ERK diminished in the control side as an embryo developed (Fig. 4A-C), in the experimental side, the activation zone of ERK remained wide (Fig. 4H).

Co-transfection of *sprouty2* and *sprouty2-DN* showed that Sprouty2-DN canceled the effects of Sprouty2. Co-transfection resulted in slight widening of the ERK activation zone (Fig. 4I). This indicates that Sprouty2-DN really suppress Sprouty2 activity.

The results indicate that Sprouty2 repressed ERK phosphorylation, and that repression of Sprouty2 activity raised the activation level of ERK. Thus, our study has confirmed that Sprouty2 functions as a negative regulator of the Ras-ERK pathway.

## Fate change of the presumptive metencephalon by Sprouty2 misexpression

Recently, It was shown that *Fgf8b* could change the fate of the presumptive mesencephalon from the optic tectum to the

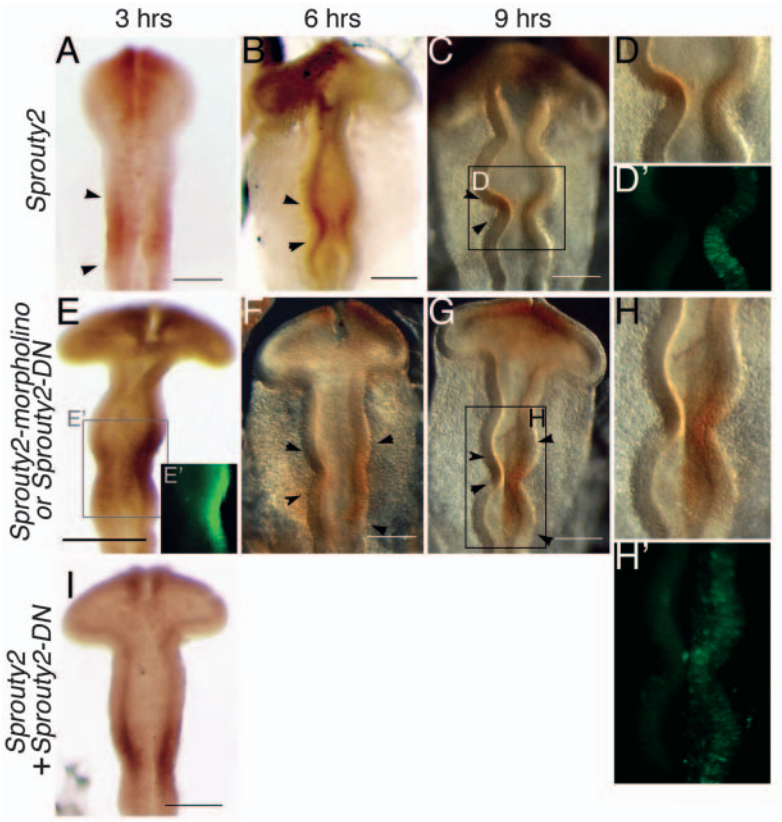


**Fig. 4.** Function of Sprouty2 as a repressor of ERK activity. (A-D) Repression of ERK activity by *sprouty2* misexpression. (E) Upregulation of ERK activity by morpholino antisense oligonucleotide against *sprouty2*, and (F-H) by misexpression of the dominant negative form of *sprouty2*. (I) Co-transfection of *sprouty2* and *sprouty2-DN*. The effects of Sprouty2 were canceled by Sprouty2-DN. Immunohistochemical staining with anti-dpERK (brown). D′, H′ and E′ are fluorescences of GFP and FITC, respectively, to indicate the misexpression site. (A,E,I) 3 hours after electroporation, (B,F) 6 hours after electroporation, (C,D,G,H) 9 hours after electroporation. G and H are higher magnifications of the isthmus region. Arrowhead indicates ERK activation zone. Scale bar: 200 μm.

cerebellum by activating the Ras-ERK signaling pathway (Sato et al., 2001; Liu et al., 2003; Sato and Nakamura, 2004). Disruption of the Ras-ERK pathway by the dominant negative form of Ras resulted in a fate change of the presumptive metencephalon to the tectum (Sato and Nakamura, 2004). We carried out misexpression of *sprouty2*, wondering whether Sprouty2 could also change the presumptive metencephalon to the mesencephalic property

We could distinguish the cerebellum and the tectum of E12.5 (HH 38) gross morphologically, because cerebellar swelling is characterized by sulci on its surface and the tectal swelling is smooth and larger than the former (Fig. 5A-C; *n*=7/10). Histologically, the cerebellum is characterized by the external granular layer, while the tectum has its distinct layer formation (Fig. 5D,E,H). After *sprouty2* misexpression, the swelling in the metencephalic region on the experimental side did not have sulci, and looked smooth (Fig. 5A,C; *n*=3/3). Histologically, the swelling did not have an external granular layer, but had the laminar structure that was comparable to the tectum proper (Fig. 5D,F,G). Thus we conclude that the optic tectum differentiated in place of the cerebellum by *sprouty2* misexpression. Trochlear nerve trajectory also supports the idea that the ectopic tectum differentiated in the metencephalic region. In normal embryos, the trochlear nucleus occupies the

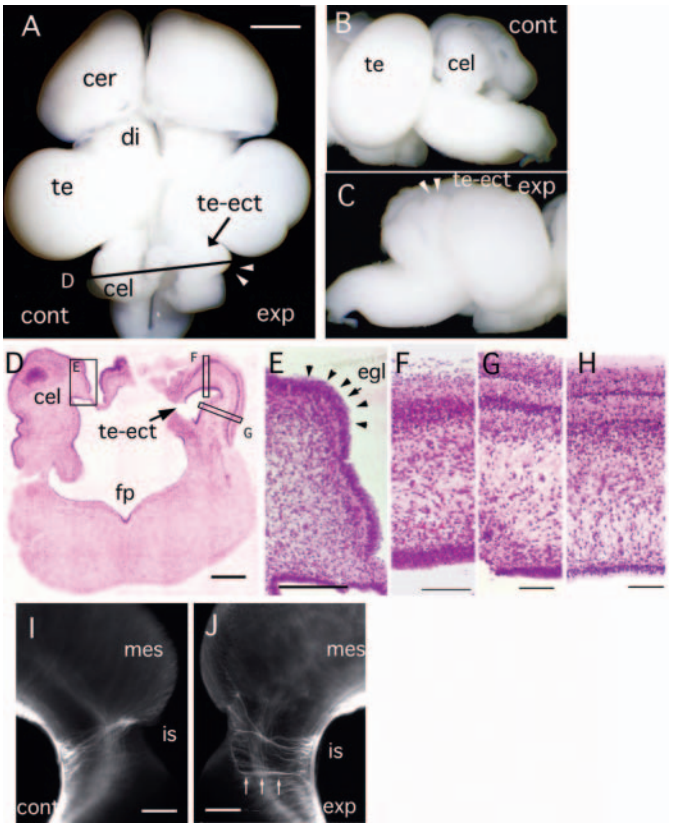


**Fig. 5.** Fate change of the presumptive cerebellum to the tectum. Dorsal view (A), control side (B), and experimental side (C) of the brain of E12.5 (HH 38) after electroporation with Sprouty2 expression vector around HH 9. (D) Horizontal section at the line indicated on A. Higher magnification of the area indicated on D (E,F,G), and the proper tectum (H). (I,J) Whole mount immunohistochemistry with anti-neurofilament antibody on HH 21 embryo, 3A10, to show trochlear nerve trajectory. After Sprouty2 misexpression, the swelling in the metencephalic region looks smooth (A,C). Histologically, the cerebellum has an external granular layer (E). The swelling on the experimental side does not have external granular layer, but has laminar structure characteristic of the tectum (compare F, G and H). A nerve bundle that resembles the trochlear nerve is added caudal to the ectopic swelling (arrows, J). cer; cerebrum; di, diencephalon; te, tectum; is, isthmus; cel, cerebellum; te-tect, tectum ectopically differentiated in the metencephalon; egl, external granular layer; fp, floor plate; exp, experimental side; cont, control side. Scale bar: 4 mm in A, 200 μm in D-J.

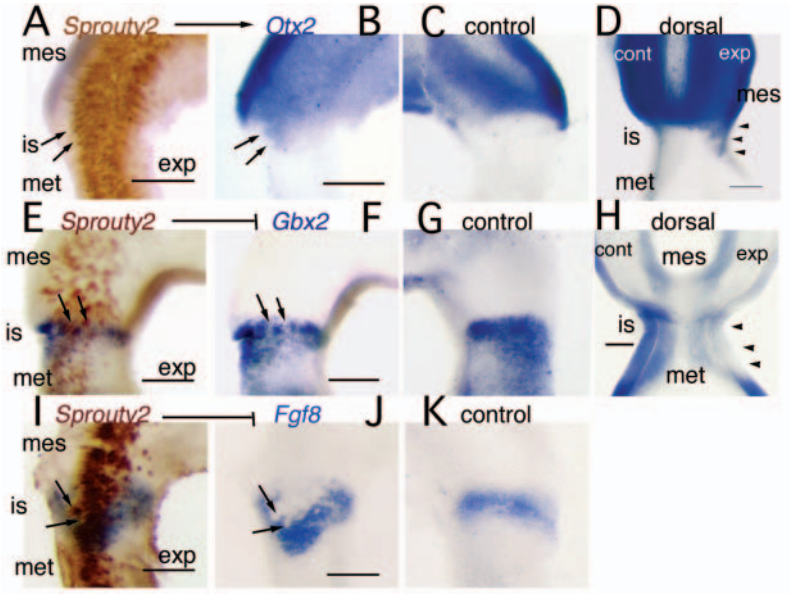


**Fig. 6.** Effects of Sprouty2 misexpression on *Otx2, Gbx2* and *Fgf8* expression. In situ hybridization for *Otx2* (A-D, purple), *Gbx2* (E-H, purple) and *Fgf8* (I-K, purple). Misexpression of Sprouty2 is assessed by immunohistochemistry with the antibody against HA-tag (A,E,I, brown); 24 hours after electroporation (HH 18; A-C,E-G,I-K); 48 hours after electroporation (HH 22; D,H). Sprouty2 induced *Otx2* expression, and repressed *Gbx2* expression in the metencephalic region. (D,H) By 48 hours after electroporation, regulation of *Otx2* and *Gbx2* expression may have occurred and the expression domain of these genes reduced. (J) The *Fgf8* expression ring shifted caudally. mes, mesencephalon; is, isthmus; cont, control side; exp, experimental side. Scale bar: 200 μm.

ventral part of the isthmus, from which nerve fibers run dorsally along the posterior margin of the mesencephalon (Fig. 5I). In the embryos at HH 21 after *sprouty2* misexpression, nerve fibers that ran along the posterior margin of the ectopic swelling could be detected in addition to the proper trochlear nerve fibers (Fig. 5J).

## Effects of Sprouty2 on the isthmus-related gene expression

We looked at the effects of Sprouty2 on mesencephalon- and metencephalon-related gene expression. It has been shown by misexpression study that the mesencephalon-metencephalon boundary is determined by repressive interaction between *Otx2* and *Gbx2* (Broccoli et al., 1999; Millet et al., 1999; Katahira et al., 2000). *Otx2* could change the fate of the metencephalic alar plate to the tectum. It was also shown that *Fgf8* expression is induced at the boundary of the *Otx2* and *Gbx2* expression domain overlapping with the *Gbx2* domain. At 24 hours after electroporation of *sprouty2* (HH 18), *Otx2* expression was induced in the metencephalic region (Fig. 6A-C; *n*=12/16). *Gbx2* expression in the metencephalic region was repressed as in the case of *Otx2* misexpression (Fig. 6E-G; *n*=7/10). By 48 hours after electroporation (HH 22), regulation of *Otx2* and *Gbx2* expression may have occurred and their expression became complementary in the metencephalic region (Fig. 6D,H; *Otx2*; *n*=6/7, *Gbx2*; *n*=3/4), which corresponds well with the morphological change observed at a later stage.

*Fgf8* is expressed in the isthmus in a ring-like pattern in normal embryos (Fig. 6K). After *sprouty2* misexpression, the *Fgf8* expression belt shifted to the caudal region (Fig. 6I,J; *n*=5/8).

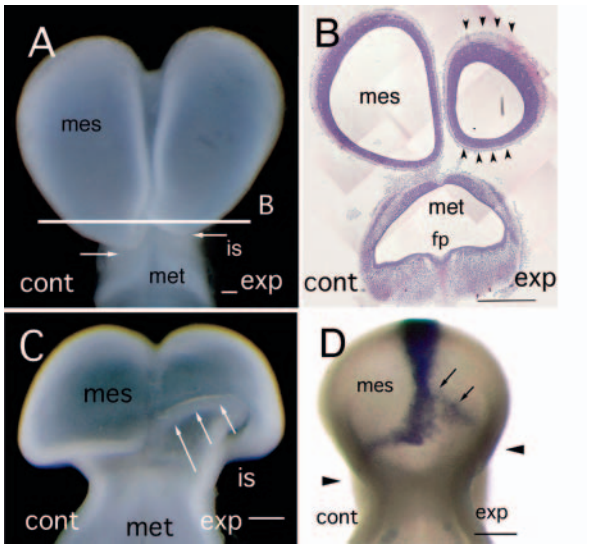


**Fig. 7.** Sprouty2-DN causes rostral shift of the caudal border of the tectum. (A,C,D) Dorsal view (A: HH 30; C: HH24; D: HH23). (B) Horizontal section of the level indicated by white line on A.(D) Whole mount in situ hybridization for *Wnt1*. Arrows indicate the caudal border of the tectum. The caudal border of the tectum at the experimental side locates rostrally to that at the control side. mes: mesencephalon, is: isthmus, met: metencephalon, fp, floor plate; cont, control side; exp, experimental side. Scale bar: 200 μm.

## Rostral shift of the isthmus by *sprouty2-DN* misexpression

Next, we carried out misexpression of *sprouty2-DN*, to see if it exerts opposite effects to *sprouty2*. As in the case of *Gbx2* misexpression, *sprouty2-DN* caused a rostral shift of the caudal boundary of the tectum (Fig. 7; *n*=5/8). The histology and expression pattern of *Wnt1* all support the rostral shift of the caudal boundary of the tectum (Fig. 7B,D; *n*=2/3). *Wnt1* is normally expressed at the posterior margin of the mesencephalon in addition to the dorsal midline of the mesencephalon. After *sprouty2-DN* misexpression, the *Wnt1* expression ring shifted rostrally (Fig. 7D).

*sprouty2*-DN induced *Gbx2* expression (Fig. 8D-F; *n*=12/13). As in the case of *Gbx2* misexpression (Katahira et al., 2000), *Otx2* expression was repressed (Fig. 8A-C; *n*=7/8). *Otx2* and *Gbx2* expression may have been regulated and their expression became complementary by 48 hours after electroporation (Fig. 8C,F; *Gbx2*; *n*=5/5, *Otx2*; *n*=3/5). *Fgf8*

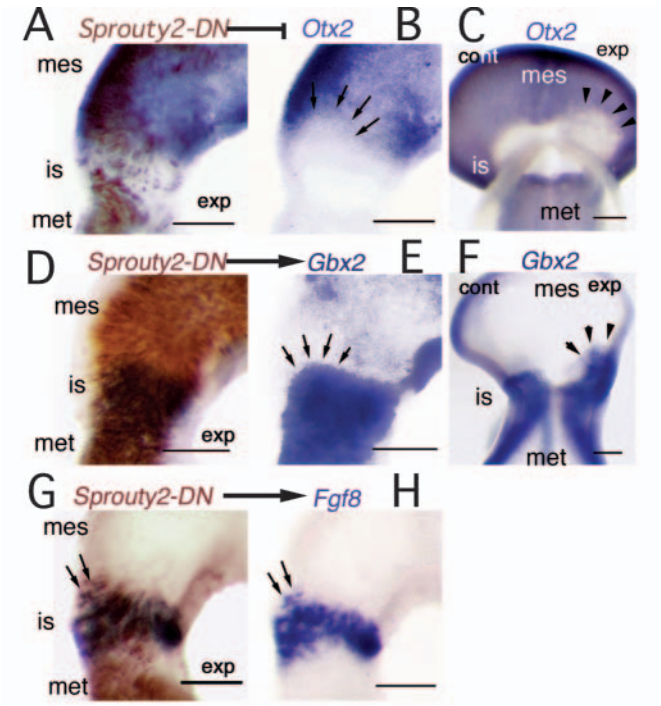


**Fig. 8.** Effects of Sprouty2-DN misexpression on *Otx2, Gbx2* and *Fgf8* expression. In situ hybridization for *Otx2* (A-C), *Gbx2* (D-F) and *Fgf8* (G,H). Misexpression of *sprouty2-DN* is assessed by immunohistochemistry with the antibody against HA-tag (A,D,G), 24 hours after electroporation (HH 18; A,B,D,E,G,H), 48 hours after electroporation (HH 22; C,F). Sprouty2-DN repressed *Otx2* expression, and induced *Gbx2* expression in the mesencephalic region. By 48 hours after electroporation, regulation of *Otx2* and *Gbx2* expression may have occurred, and the expression domain of these genes abuts (C,F). The *Fgf8* expression ring is extended rostrally (arrows on G,H). mes, mesencephalon; is, isthmus; met, metencephalon; cont, control side; exp, experimental side. Scale bar: 200 μm.

expression was induced at the caudal part of the mesencephalon (Fig. 8G,H).

## Discussion

In the present study we have shown that: (1) Sprouty2 interferes with ERK activation, (2) *sprouty2* is induced very rapidly by both Fgf8a and Fgf8b, (3) after *Fgf8b* misexpression that causes fate change of the presumptive mesencephalon to the metencephalon, endogenous *Fgf8* expression is induced along the roof plate, and Sprouty is also induced overlapping the new *Fgf8* expression site, (4) *sprouty2* misexpression changes the fate of the presumptive metencephalon to the mesencephalon, (5) a dominant negative form of Sprouty2 activates ERK and causes anterior shift of the posterior margin of the tectum.

### Sprouty2 is induced by Fgf8 but negatively regulates the Fgf8-Ras-ERK signaling pathway

In normal development, ERK activation was seen at the site of Fgf8 expression, that is, the anterior neural ridge and the isthmus. It has been indicated that Sprouty2 acts as a negative feedback regulator of the growth factor-mediated Ras-ERK signaling pathway (Hacohen et al., 1998; Kramer et al., 1999; Casci et al., 1999; Lin et al., 2002). On the other hand, contrary effects of Sprouty were reported for EGF receptor (EGFR)-mediated signaling (Egan et al., 2002; Wong et al., 2002; Fong et al., 2003; Hall et al., 2003; Rubin et al., 2003; Stang et al., 2004). hSprouty2 sequestrated c-Cbl, which in turn abrogated EGFR ubiquitylation and endocytosis, and consequently sustained EGF-induced ERK signaling. The present study showed that misexpression of Sprouty2 interfered with ERK activation, and Sprouty2-DN augmented ERK activation as assessed by staining with anti-diphosphorylated ERK. Morpholino antisense oligonucleotide against Sprouty2 also augmented activity of ERK. Thus we have concluded that Sprouty2 negatively regulates the Ras-ERK pathway in the isthmus.

*sprouty2* was induced by both Fgf8a and Fgf8b. Induction of *sprouty2* by Fgf8 is very rapid. *sprouty2* induction could be already seen by 3 hours after electroporation of both Fgf8a and Fgf8b corresponding to the *Fgf8* misexpression site. If we consider that the translation products could be detectable by 2 hours after electroporation, *sprouty2* induction may occur within an hour of Fgf8 expression. The results indicate that the Fgf8b signal is transduced very rapidly via the Ras-ERK signaling pathway, and controls transcription of the downstream gene.

### Regulation of the organizing center

*Fgf8b* misexpression by electroporation at a concentration of 0.1 μg/μl or 1 μg/μl changes the fate of the presumptive mesencephalon to that of the metencephalon (Sato et al., 2001). After 24 hours of electroporation of 1.0 μg/μl of *Fgf8b* vector, *Fgf8* and S*prouty2* expression was observed on the lateral side of the mesencephalon and along the roof plate, while expression along the roof plate did not exist after *Fgf8a* misexpression. Liu et al. (Liu et al., 2003) suggested that misexpression of *Fgf8* along the roof plate represents new isthmus after *Fgf8* misexpression, that is, *Fgf8* expression along the roof plate is from the embryonic gene. We tried to confirm this notion by electroporating mouse *Fgf8b* expression vector, and by hybridization in situ with chick and mouse *Fgf8b* probes that do not cross hybridize each other. It was revealed that *Fgf8* expression along the roof plate is the transcript from the endogenous chick *Fgf8,* and that *Fgf8* mRNA on the lateral side of the mesencephalon was that transcribed from the introduced mouse *Fgf8b*. Since expression along the roof plate did not exist after *Fgf8a* misexpression, we supposed that new isthmus is formed along the roof plate, and consequently, mesencephalon may have acquired the metencephalic property after *Fgf8b* misexpression. After electroporation with 0.1 μg/μl Fgf8b expression vector, which also changes the fate of the presumptive mesencephalon to the metencephalon, new isthmus was set as V-shaped. It also indicates that *sprouty2* is induced at the ectopic site very rapidly and negatively regulates Fgf signaling.

It was shown that *Fgf8* is induced at the interface of the *Otx2* and *Gbx2* expression domain, overlapping with *Gbx2* expression (Broccoli et al., 1999; Millet et al., 1999; Hidalgo-Sanchez et al., 1999; Katahira et al., 2000; Garda et al., 2001). After *Fgf8b* misexpression, a new *Fgf8* expression line is set at the border of the *Otx2* and *Gbx2* expression domain.

Reconstruction of the *Fgf8* expression line may be a result of interaction among Fgf8, Limx1b, Wnt1, Otx2 and Gbx2 (Garda et al., 2001; Matsunaga et al., 2002). It was shown that Limx1b represses *Fgf8* expression in a cell-autonomous fashion (Matsunaga et al., 2002), Limx1b induces *Wnt1* expression, and Wnt1 in turn induces *Fgf8* expression. Thus Limx1b induces *Fgf8* expression around Limx1b-expressing cells. Although Gbx2 represses *Limx1b* expression, Fgf8 induces *Limx1b* expression (Matsunaga et al., 2002). After Fgf8b misexpression, Limx1b expression was induced in the mesencephalic region in a similar pattern to *Fgf8* misexpression at first. Then Limx1b may have repressed endogenous *Fgf8* expression in the isthmus. Since Fgf8b induces *Gbx2*, *Limx1b* expression in the mesencephalic region may have been repressed by Gbx2, and disappeared by 36 hours after electroporation. Finally, ring-like expression remains rostral to the *Gbx2* expression. Now the roof plate has become the interface of *Otx2* and *Gbx2* expression in the mesencephalic region, *Fgf8* expression may have been induced along the roof plate.

### Negative feedback regulation of isthmus organizing activity by Sprouty2

As discussed before, Fgf8b could change the property of the presumptive mesencephalon to that of the metencephalon. This raises a question as to how the Fgf8 signal is transduced to organize cerebellar differentiation. This subject was challenged by disrupting the Ras-ERK signaling pathway by misexpression of dominant negative form of Ras (Sato and Nakamura, 2004). Since disruption of the Ras-ERK signaling pathway resulted in differentiation of the optic tectum in place of the cerebellum, and Ras-DN canceled the effects of Fgf8b after co-electroporation of Fgf8b and Ras-DN, it was suggested that Fgf8b activates the Ras-ERK signaling pathway to organize cerebellar differentiation. Very recent study showed that Ras-ERK signaling cascades modulate the activity of Irx2 by phosphorylation for the cerebellar development (Matsumoto et al., 2004).

It was reported that Sprouty2 negatively regulates the Ras-ERK signaling pathway. We have shown that Sprouty2 really repressed ERK phosphorylation. Misexpression of Sprouty2 induced *Otx2* expression and repressed *Gbx2* expression in the metencephalon, and resulted in the differentiation of tectum in place of the cerebellum. As in the case of the dominant negative form of *Ras* misexpression, the swelling in the metencephalic region after *sprouty2* misexpression did not show cerebellar sulci on its surface but was smooth. Histologically, it did not contain the external granular layer, which is characteristic of the cerebellum, but consisted of the laminar architecture characterisitic of the tectum. Misexpression of *sprouty2-DN* exerted the reverse effects to *sprouty2*. *Gbx2* was induced in the mesencephalon by Sprouty2-DN, and the obtained results were similar to those after Gbx2 misexpression. On the control side, ERK activation zone in the isthmus narrows from stage 9 to 10 (see Fig. 4A-C,F,G). Repression of Sprouty2 activity by misexpression of Sprouty2-DN or by application of morpholino antisense oligonucleotide, interfered with narrowing of the ERK activation zone (see Fig. 4E-H). The results indicate that the Ras-ERK signal should be weakened in a short period of development, and Sprouty2 may contribute to weakening of the Ras-ERK signaling. Taken together, the results indicate that the Fgf8 signal is so strong that a negative regulator is needed. Once the Fgf8 signal is transduced, it quickly induces its negative regulator, thus the negative feedback loop may regulate the Fgf8 signaling.

Recently, another negative regulator for Fgf signaling, Sef, was reported (Minowada et al., 1999; Fürthauer et al., 2002; Lin et al., 2002). *Sef* is expressed in an overlapping manner to *Fgf8*, induced by Fgf8, and functions as a negative regulator for Fgf8 signaling. *Sef* is also expressed in the isthmus (Fürthauer et al., 2002; Lin et al., 2002). The relationship between Sef and Sprouty must be elucidated, but the Fgf8 signaling plays a crucial role in morphogenesis and is so strong, its signaling may be regulated repeatedly.

In conclusion, Fgf8 activates the Ras-ERK signaling pathway, and very rapidly regulates downstream gene expression. *sprouty2* is induced very rapidly by Fgf8-Ras-ERK signaling, and regulates this pathway negatively for the metencephalon to receive appropriate signaling. We have shown that regionalization of the neural tube is disturbed by both hyper- and hypo-signaling.

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