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Activation of Ras-ERK pathway by Fgf8 and its downregulation by Sprouty2 for the isthmus organizing activity

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ABSTRACT

In the previous studies, we showed that strong Fgf8 signaling activates the Ras-ERK pathway to induce cerebellum. Here, we show importance of negative regulation following activation of this pathway for proper regionalization of mesencephalon and metencephalon in chick embryos. 'Prolonged' activation of ERK by misexpression of *Fgf8b* and dominant-negative *Sprouty2* (*dnSprouty2*) did not change the fate of the mesencephalic alar plate. Downregulation of ERK activity using an MEK inhibitor, U0126, or by tetracycline-dependent Tet-off system after co-expression of *Fgf8b* and *dnSprouty2* forced the mesencephalic alar plate to differentiate into cerebellum. We then paid attention to Mkp3. After misexpression of *dnMkp3* and *Fgf8b*, slight downregulation of ERK activity occurred, which may be due to Sprouty2, and the mesencephalon transformed to the isthmus-like structure. The results indicate that ERK must be once upregulated and then be downregulated for cerebellar differentiation and that differential ERK activity level established by negative regulators receiving Fgf8 signal may determine regional specificity of mesencephalon and metencephalon.

Introduction

Vertebrate brain is first designed as primary brain vesicles; prosencephalon, mesencephalon and rhombencephalon, which then subdivided into secondary brain vesicles; telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon. Classic transplantation experiments revealed that mes–metencephalic boundary (isthmus) functions as an organizer for the mesencephalon and metencephalon (Martinez et al., 1991; Nakamura, 1990; Nakamura et al., 1986). Alar diencephalon differentiates into the tectum when transplanted to the posterior mesencephalon but keeps its original fate when transplanted at the anterior mesencephalon (Nakamura et al., 1986). Isthmus could induce tectum in the diencephalon (Martinez et al., 1991). *Fgf8* is expressed in the isthmus and could mimic isthmus activity when transplanted into the diencephalon (Crossley et al., 1996). Now it is accepted that Fgf8 acts as an organizer for the mesencephalon and metencephalon (Crossley et al., 1996; Garda et al., 2001; Liu et al., 1999; Martinez et al., 1999; Nakamura, 2001; Nakamura et al., 2008; Sato et al., 2001).

Among 8 splicing isoforms of *Fgf8*, *Fgf8a* and *Fgf8b* are expressed in the isthmus (Sato et al., 2001). Fgf8b conveys stronger signal than Fgf8a since Fgf8b binds FgfR with higher affinity (Olsen et al., 2006). Misexpression of *Fgf8b* transforms the mesencephalon to metencephalon while *Fgf8a* misexpression transforms diencephalon to mesencephalon. Difference in the effects could be ascribed to the difference in the signal strength since misexpression of *Fgf8b* at hundredfold lower concentration exerted Fgf8a type effects (Sato et al., 2001). Since disruption of Ras-ERK signaling by misexpression of dominant-negative form of *Ras* changed the fate of the metencephalon from the cerebellum to tectum, it has been accepted that strong Fgf8 signal activates the Ras-ERK signaling pathway to organize cerebellar differentiation (Sato et al., 2001, 2004; Sato and Nakamura, 2004). On the other hand, there are several negative regulators for Ras-ERK signaling pathway, which are expressed overlapping to *Fgf8* (Casci et al., 1999; Chambers and Mason, 2000; Echevarria et al., 2005; Furthauer et al., 2002; Hacohen et al., 1998; Kawakami et al., 2003; Lin et al., 2002; Mason et al., 2006; Smith et al., 2005; Suzuki-Hirano et al., 2005; Tsang et al., 2002; Zhang et al., 2001). *Sprouty2* is one of them (Basson et al., 2008; Casci et al., 1999; Chambers and Mason, 2000; Hacohen et al., 1998; Jukkola et al., 2006; Lin et al., 2002; Liu et al., 2003; Mason et al., 2006; Suzuki-Hirano et al., 2005; Zhang et al., 2001). *Sprouty2* is induced by Fgf8, but it downregulates ERK activity (Mason et al., 2006; Suzuki-Hirano et al., 2005).

Importance of regulation of Ras-ERK signaling has been shown in many systems. Sustained activation of ERK leads PC 12 cells to differentiation to sympathetic neurons, while transient activation of

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ERK leads to proliferation (Marshall, 1995), which indicates that duration of ERK activation is crucial for the cellular response. It was shown that negative regulation of Fgf8 signaling by Sprouty2 is indispensable for the differentiation of the organ of Corti of mice (Shim et al., 2005) and for proper regionalization of the mesencephalon and metencephalon (Basson et al., 2008; Nakamura et al., 2008; Suzuki-Hirano et al., 2005). Upregulation of Ras-ERK signaling pathway by misexpression of dominant-negative form of *Sprouty2* (*dnSprouty2*) caused anterior shift of the mes–metencephalon boundary (Suzuki-Hirano et al., 2005). On the other hand, down-regulation of Ras-ERK pathway by misexpression of *Sprouty2* caused posterior shift of the mes–metencephalic boundary to change the fate of the metencephalon to the tectum (Suzuki-Hirano et al., 2005). *Fgf8* gene dose-dependent differentiation of mesencephalon to rhombomere 1 (r1) was reported recently by conditional misexpression of *Sprouty2* in mice (Basson et al., 2008). Mice that misexpress *Sprouty2* in mesencephalon-r1 showed increase in cell death in the anterior mesencephalon and cell fate change of the posterior mesencephalon to that of the anterior mesencephalon. In the r1, vermis was affected by conditional *Sprouty2* misexpression (Basson et al., 2008).

In normal development, ERK is phosphorylated widely in the mes–metencephalic region around stages 8 and 9 (Hamburger and Hamilton, 1951; Sato and Nakamura, 2004; Suzuki-Hirano et al., 2005). ERK phosphorylation region becomes narrower, and ERK phosphorylation level in the metencephalon becomes very low by stage 12 (Sato and Nakamura, 2004; Suzuki-Hirano et al., 2005). Reexamination of the mesencephalon after Fgf8b misexpression, which would differentiate to the cerebellum, revealed that ERK was first activated and that the activity was downregulated by 18 h after electroporation. So we speculated that ERK should be once activated and then be downregulated by negative regulators for the Ras-ERK signaling for cerebellar differentiation. In order to assess our speculation, we first kept ERK phosphorylation level high by co-electroporation of *Fgf8b* with *dnSprouty2* (dominant-negative form of *Sprouty2*) expression vector. Next we downregulated ERK activity after co-transfection of *Fgf8b* and *dnSprouty2* by inserting a bead soaked in U0126, an MEK inhibitor, or by turning off transcription of *dnSprouty2* by tetracycline-controlled method (Hilgers et al., 2005; Sato et al., 2007). We obtained results to support that both magnitude and duration of ERK activation is indispensable for cerebellar differentiation; that is, once activated, ERK should be downregulated for cerebellar differentiation. We also carried out misexpression of *Fgf8b* and *dnMkp3* and came to the conclusion that the differential activation level of ERK is established by negative regulators, and according to the ERK activation level proper regionalization of the midbrain and hindbrain may occur. Sprouty2 may be indispensable for establishing the region-specific ERK activation pattern.

Materials and methods

In ovo electroporation

In ovo electroporation of expression vectors was carried out at stages 8 and 9 (5–7 somite stages; Hamburger and Hamilton, 1951) as described previously (Funahashi et al., 1999; Nakamura and Funahashi, 2001; Odani et al., 2008). GFP expression vector (pCA-GAP-GFP) was co-electroporated to ascertain the efficiency of transfection. GFP was detected by whole-mount immunostaining with anti-GFP antibody or *in ovo* imaging. Dominant-negative form of *Sprouty2* (*dnSprouty2*) was created by substituting alanine for tyrosine 55 of *Sprouty2* (Sasaki et al., 2001). *Fgf8b* and *dnSprouty2* were inserted in pMiwIII as described previously (Sato et al., 2001; Suzuki-Hirano et al., 2005). Since pMiwIII has Rous sarcoma virus enhancer and chick β-actin promoter, it assures ubiquitous expression of the transgene (Matsunaga et al., 2001; Suemori et al., 1990; Wakamatsu et al., 1997). Dominant-negative form of *Mkp3 (dnMkp3)* in pCS2, provided by Dr. Kawakami (Kawakami et al., 2003), was cut out and re-inserted in pMiwIII vector for electroporation.

Cells that misexpressed *dnSprouty2* and *Fgf8b* were traced of their fate on E10.5 by transposon mediated genome integration of *GFP*. For this, pCAGGS-T2TP (0.66 μg/μl) (kind gift of Prof. Takahashi; Watanabe et al., 2007) and pT2K-CAGGS-GFP (0.66 μg) (Harada et al., 2008) were electroporated along with pMiwIII-Fgf8b (1 μg/μl)/ pMiwIII-dnSprouty2 (2 μg/μl) mixture to stages 8 and 9 embryos.

One may raise a question if co-electroporated transgenes are really expressed in the same cell. To answer this question, efficiency of co-expression of co-electroporated genes was checked by electroporating pT2K-CAGGS-mCherry-Nuc (2.00 μg/μl, constructed by Dr. Y. Watanabe) and pT2K-CAGGS-GFP (0.66 μg/ μl, one third of pT2K-CAGGS concentration). At 36 h after electroporation ($n=12$; 3 sections of each 4 embryos), $98.74\pm0.31\%$ of the GFP expressing cells co-expressed mCherry-Nuc, indicating that almost all the GFP expressing cells express transgene.

Histology

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

Bead implantation

A bead soaked in U0126 (Wako), an MEK1/2 inhibitor, was implanted into the mesencephalic region 17 h after electroporation. U0126 was diluted in DMSO to a final concentration of 10 mM, where Affigel Blue beads (Bio-Rad) or AG1-X2 ion-exchange resin beads (Bio-Rad) were placed as described previously (Sato and Nakamura, 2004).

Tet-off

HA-tagged *dnSprouty2* (Suzuki-Hirano et al., 2005) was inserted in TRE-driven BI-EGFP vector (pBI-EGFP-dnSpry2, Constructed by Dr. Nakagawa, and we got license from Clonetec) (Watanabe et al., 2007). pBI-EGFP-dnSpry2, pCAG-tTA (Tet-controlled transcriptional activator) (Tet-OFF), pCAGGS-T2TP and pCAGGS-Fgf8b were co-electroporated at HH 8 embryos. The embryos were administered with 0.2 mg/ml Doxycyclin (Dox, Clonetech) in PBS at 5.5 h after co-electroporation, and then Dox was administered at every 24 h. We confirmed that *dnSpry2* was expressed at 6 h after co-electroporation and was not expressed by 12 h after DOX administration by immunostaining for HA-tag (data not shown).

In situ hybridization

In situ hybridization was carried out according to the method as described previously (Suzuki-Hirano et al., 2005), which is a modification of Wilkinson (Wilkinson, 1992). Probes for *Otx2, Gbx2, Fgf8* and *Mkp3* are described in Katahira et al. (2000) or in Kawakami et al. (2003).

Immunohistochemistry

For immunohistochemistry, anti-GFP rabbit polyclonal antibody (Molecular Probe), anti-diphosphorylated ERK antibody, (Sigma or Cell Signaling Technology), anti-neurofilament antibody (3A10, DSHB) and anti-Pax6 antibody (DSHB) were used as primary antibodies. As secondary antibodies, Alexa488-conjugated anti-rabbit IgG, Alexa594-conjugated anti-rabbit IgG, Alexa594-conjugated anti-mouse IgG (Molecular Probe), biotinylated anti-mouse IgG antibody (VECTOR) and horseradish peroxidase (HRP)-conjugated anti-mouse