Development 129, 5269-5277 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV1828

Role of Lmx1b and Wnt1 in mesencephalon and metencephalon development

Eiji Matsunaga*, Tatsuya Katahira and Harukazu Nakamura†

Department of Molecular Neurobiology, Institute of Development, Aging & Cancer, and Graduate School of Life Sciences, Tohoku University, Seiryo-machi 4-1, Aoba-ku, Sendai 980-8575, Japan

*Present address: INSERM U106, Bâtiment de Pédiatrie, Hôpital de la Salpêtrière, 47 Boulevard de l'Hôpital, 75013 Paris, France
†Author for correspondence (e-mail: nakamura@idac.tohoku.ac.jp)

Accepted 12 August 2002

SUMMARY

The isthmus is the organizing center for the tectum and cerebellum. Fgf8 and Wnt1 are secreted molecules expressed around the isthmus. The function of Fgf8 has been well analyzed, and now accepted as the most important organizing signal. Involvement of Wnt1 in the isthmic organizing activity was suggested by analysis of *Wnt1* knockout mice. But its role in isthmic organizing activity is still obscure. Recently, it has been shown that *Lmx1b* is expressed in the isthmic region and that it may occupy higher hierarchical position in the gene expression cascade in the isthmus. We have carried out misexpression experiment of *Lmx1b* and *Wnt1*, and considered their role in the isthmic organizing activity. *Lmx1*b or *Wnt1* misexpression caused expansion of the tectum and cerebellum. *Fgf8* was repressed in a cells that misexpress *Lmx1b*, but *Fgf8* expression was induced around *Lmx1b*-misexpressing cells. As Lmx1b induced *Wnt1* and Wnt1 induced *Fgf8* expression in turn, Wnt1 may be involved in non cell-autonomous induction of *Fgf8* expression by Lmx1b. Wnt1 could not induce *Lmx1b* expression so that *Lmx1b* may be put at the higher hierarchical position than *Wnt1* in gene expression cascade in the isthmus. We have examined the relationship among isthmus related genes, and discuss the mechanism of the formation and maintenance of isthmic organizing activity.

Key words: Lmx1b, Wnt1, Fgf8, Isthmus, Chick

INTRODUCTION

The isthmus works as an organizing center for the tectum and cerebellum (Martinez et al., 1991; Alvarado-Mallart, 1993; Marin and Puelles, 1994; Martinez et al., 1995). *Fgf8* is one of the secreted molecules expressed in the isthmus. As Fgf8-soaked beads transplanted into the diencephalon induced an ectopic tectum or cerebellum (Martinez et al., 1995; Crossley et al., 1996; Martinez et al., 1999), *Fgf8* is thought to be the organizing molecule. This notion has further been confirmed by *Fgf8* misexpression in mice and chick in which Fgf8 caused complete fate change of the diencephalon and the mesencephalon to cerebellum (Liu et al., 1999; Sato et al., 2001).

It was shown that *Otx2* and *Gbx2* repress each other's expression to make mes-metencephalic boundary (Broccoli et al., 1999; Millet et al., 1999; Katahira et al., 2000). At the *Otx2* and *Gbx2* expression boundary, *Fgf8* is induced overlapping with *Gbx2* expression domain, which was shown in transplantation or misexpression experiments (Hidalgo-Sanchez et al., 1999; Katahira et al., 2000). In combination culture of mesencephalic and metencephalic tissue, *Fgf8* expression was induced at the boundary (Irving and Mason, 1999). As Otx2 and Gbx2 are transcription factors, involvement of secreted factor(s) or cell surface molecule(s) in Fgf8 induction is assumed.

Wnt1 is a secreted molecule and is expressed in the isthmus. *Wnt1* mutant mice show deletion in the mesencephalon and the metencephalon (McMahon et al., 1992). *Lmx1b* is one of LIM homeodomain proteins and is expressed in connection with *Wnt1*. Misexpression by the retrovirus vector showed that Lmx1b could induce *Wnt1* expression (Adams et al., 2000). Expression patterns of *Lmx1b* and *Wnt1* are well correlated with *Fgf8* expression in the isthmus region. In normal development, expression domain of *Lmx1b* and *Wnt1* and that of *Fgf8* overlaps broadly around the isthmic region in the early stage, while their expression domains become segregated and located side by side by E2.5. Therefore, we hypothesized that both Lmx1b and Wnt1 were involved in the formation and maintenance of the isthmus organizer. To explore the function of *Lmx1b* and *Wnt1* in the isthmus organizer, we carried out misexpression of *Lmx1b* and *Wnt1* by in ovo electroporation. *Lmx1b* misexpression induced *Wnt1*, *Otx2* and *Grg4*, but repressed *Fgf8* cell-autonomously. On the one hand, *Wnt1* misexpression induced *Fgf8* expression non cell-autonomously. Hence, Lmx1b represses *Fgf8* expression cell-autonomously provably via Grg4 and induced non cell-autonomously via Wnt1. On the other hand, *Fgf8* misimpression induced *Lmx1b* expression non cell-autonomously. Otx2 induced *Lmx1b* expression, while Gbx2 represses *Lmx1b* expression. Thus, cell-autonomous and non cell-autonomous regulation among Otx2, Gbx2, Fgf8, Lmx1b and Wnt1 are deeply involved in formation and maintenance of the isthmus organizer activity.

MATERIALS AND METHODS

Expression vectors

First chick *Lmx1b* cDNA was isolated as two fragments by PCR from E3 chick brain cDNA as a template. Primers for N- and C-terminal fragments are 5′-CCCATATGGACATCGCCTC-3′, 5′-AGGTCTCC-TTGGGTCCTTCC-3′ and 5′-GCTGAGAAAAGGGGATGAGT-3′, 5′-TTCATGAGGCGAAATAGGAG-3′, respectively. Primers for the N-terminal deletion (LIM domain deletion) of *Lmx1b* (*Lmx1b-C*) are 5′-GCATGAGCGATGATGAAGATGGAGA-3′ and 5′-CGAAATA-GGAGCTCTGCATA-3′ (the start codon is attached in N-terminal primer). Obtained fragments were fused at *Sac*I site to make a full length of *Lmx1b*. The *Lmx1b-EnR* is a fusion of *Lmx1b* with *En2* repressor domain and HA-tag (Matsunaga et al., 2000). The full-length chick *Wnt1* cDNA was isolated from E2 chick brain cDNA library. These fragments were inserted in pMiwIII, a derivative of pMiwSV and designated as pMiw-Lmx1b, pMiw-Wnt1, etc. (Suemori et al., 1990; Wakamatsu, 1997), which has Rous sarcoma virus enhancer and chicken β-actin promoter. *Otx2*, *Gbx2* and *Fgf8b* expression vectors have been described previously (Katahira et al., 2000; Sato et al., 2001).

In ovo electroporation

Fertilized chicken eggs from a local farm were incubated at 38ºC. For transfection, in ovo electroporation on stage 10 chick embryos (Hamburger and Hamilton, 1951) was adopted as previously described (Funahashi et al., 1999). Green fluorescence protein (*GFP*) expression vector (pEGFP-N1, Clontech) was co-electroporated to check the efficiency.

In situ hybridization

In situ hybridization for whole mount and for sections was performed as described (Bally-Cuif et al.,1995; Ishii et al., 1999). Probes for *Fgf8*, *Otx2*, *Gbx2*, *Wnt1*, *Pax2*, *Grg4* and *Cash1* have been described previously (Jasoni et al., 1994; Araki and Nakamura, 1999; Okafuji et al., 1999; Funahashi et al., 1999; Katahira et al., 2000; Sugiyama et al., 2000). For *Lmx1b* probe, the full length of *Lmx1b* was used. Digoxigenin (DIG)- or fluorescein isothiocyanate (FITC)-labeled antisense RNA was generated by T3 or T7 RNA polymerase (Funahashi et al., 1999). Alkaline phosphatase (ALP)-conjugated anti-DIG or anti-FITC sheep-polyclonal antibody (Roche Molecular Biochemicals) was used for detection. For double in situ hybridization, Fast Red TR/Naphthol AS/MX (Sigma FAST™; Sigma) was used for detection of the first signal, and 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were used for detection of the second signal. ALP for the first detection was inactivated by incubating with 100 mM glycine-HCl (pH 2.2) for about 15 minutes at room temperature. In some cases, Fast Red staining was washed out in ethanol, and NBT staining was washed out by incubating in dimethylformamide (DMF) at 55ºC.

BrdU incorporation

BrdU (Bromodeoxyuridine) solution (10 mM, Sigma) was injected into the yolk vein 48 and 72 hours after electroporation. Thirty minutes after BrdU injection, the embryos were fixed in 4% paraformaldehyde in PBS. Incorporated BrdU was detected by the addition of monoclonal anti-BrdU antibody (Roche), followed by incubation with Alexa 594-conjugated anti-mouse secondary antibody (Molecular Probes). For the quantitative analysis, BrdU-positive area was measured by Aqua Cosmos image analyzer (Hamamatsu Photonics), and corresponding area of the experimental and control side on the same section was compared.

Immunohistochemistry

Rat monoclonal anti-HA antibody (Roche Molecular Biochemicals) was used as a primary antibody. Horseradish peroxidase (HRP)-conjugated anti-rat IgG antibody (Iwaikougaku-yakuhin) was used as the second antibody.

Histology

Embryos embedded in Technovite 7100 (Kulter) were serially sectioned at 5 μm, and stained with Hematoxylin-Eosin, as previously described (Matsunaga et al., 2001).

RESULTS

Expression pattern of *Lmx1b*, *Wnt1* and *Fgf8*

We first examined spatial and temporal expression patterns of *Lmx1b*, *Wnt1* and *Fgf8*. As reported before (Yuan and Schoenwolf, 1999; Adams et al., 2000), *Lmx1b* is expressed from the diencephalon to the metencephalon at stage 9. At stage 10, *Wnt1* expression covers whole mesencephalon and the isthmus. The expression domain of *Lmx1b* is completely included in that of *Wnt1*, but a little bit narrower; *Lmx1b* is not expressed in the anterior part of the mesencephalon. *Fgf8* is expressed in the metencephalon and isthmus so that the expression domain of *Lmx1b*, *Wnt1* and *Fgf8* overlaps in the isthmic region (Fig. 1A,C,E). Overlapping region becomes gradually reduced. At stage 12, *Lmx1b* is expressed strongly in the mesencephalon, but weakly in the metencephalon, where *Fgf8* is expressed (Fig. 1B,D). Expression domain of *Wnt1* is almost segregated from that of *Fgf8* (Fig. 1F). By E2.5 (HH17), the expression domain of *Lmx1b* and *Wnt1* completely overlaps at the posterior margin of the mesencephalon, and just posterior to it expression domain of *Fgf8* is located so that the expression domain of *Lmx1b* and *Wnt1* becomes side by side to that of *Fgf8* at the mes-metencephalic boundary (Fig. 1G,H).

Morphology after *Lmx1b* or *Wnt1* misexpression

From the spatial and temporal expression pattern of *Lmx1b*, *Wnt1* and *Fgf8*, we suspected that they may regulate each other's expression in the isthmic region and may play a role in organizing activity. It has already been reported that *Lmx1b* and *Wnt1* play important roles in maintenance of the isthmic organizing activity by misexpression with retrovirus vectors (Adams et al., 2000). We adopted in ovo electroporation for misexpression, because in ovo electroporation assures more rapid and stronger misexpression.

First, we carried out *Lmx1b* misexpression experiment. By E7.5 (6 days after electroporation of pMiw-Lmx1b), the size of the tectum was expanded (n=8/8) (Fig. 2A-C). Torus semicircularis was also expanded (n=2/2) (Fig. 2C). The rhombic lip, which is a primordium of the cerebellum, expanded to the posterior (Fig. 2B,C). These results indicate that both mesencephalon and metencephalon are enlarged by *Lmx1b* misexpression.

It has been reported that *Wnt1* misexpression with retrovirus vectors had not affected tectum development (Adams et al., 2000). As transfection by electroporation exerts more drastic effects than retrovirus system (Nakamura and Funahashi, 2001), we carried out electroporation with pMiw-Wnt1. *Wnt1* misexpression resulted in expansion of the mesencephalon by 48 hours after electroporation (Fig. 2D,E). At E14.5 (13 days after electroporation), the telencephalon, the tectum and the cerebellum were all enlarged. In the cerebellum some extra folia were formed (n=3/5) (Fig. 2F-H).

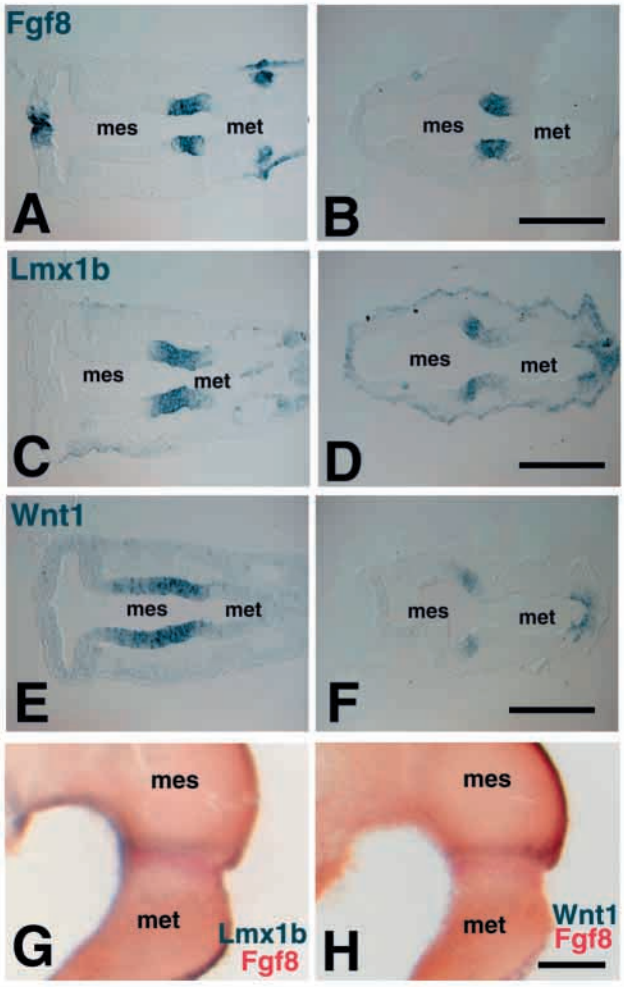

Fig. 1. Normal expression patterns of *Fgf8*, *Lmx1b* and *Wnt1*. In situ hybridization of serial sections of the same embryos at stage 10 (A,C,E) and stage 12 (B,D,F), for *Fgf8* (A,B), *Lmx1b* (C,D) and *Wnt1* (E,F). At stage 10, *Lmx1b* and *Wnt1* are expressed in the mesencephalon and metencephalon. Their expression overlaps with *Fgf8* expression in the metencephalon. At stage 12, *Lmx1b* is expressed strongly in the mesencephalon, but in the metencephalon its expression is weak. *Wnt1* expression in the metencephalon has almost disappeared. (G,H) Whole-mount in situ hybridization for *Lmx1b* (blue) and *Fgf8* (red) (G), and for *Wnt1* (blue) and *Fgf8* (red) (H). Both *Lmx1b* and *Wnt1* are expressed next to *Fgf8* expression at the mes-metencephalic boundary. Scale bars: 250 μm. mes, mesencephalon; met, metencephalon.

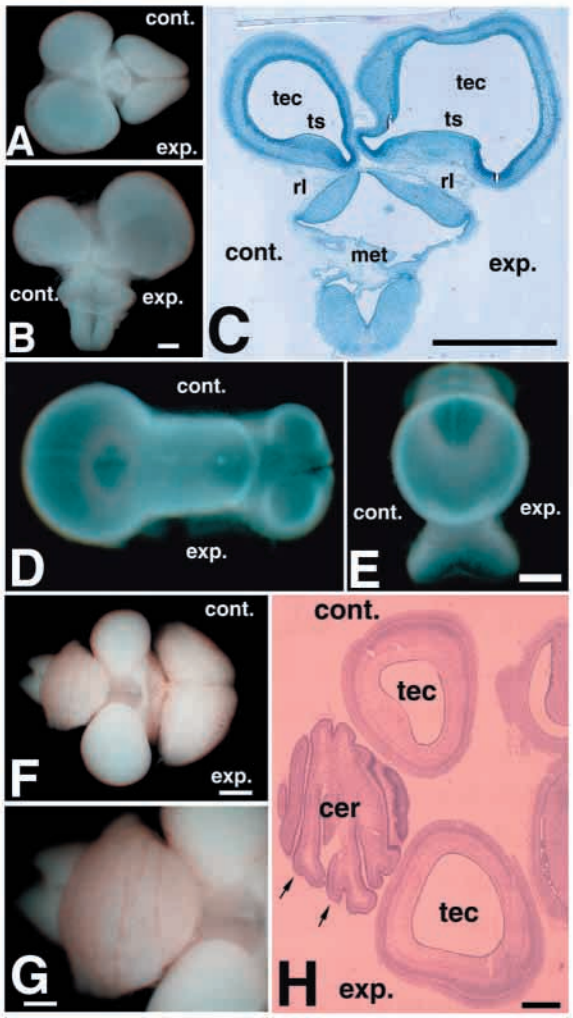

Fig. 2. Morphology after *Lmx1b* and *Wnt1* misexpression. (A-C) Morphology of *Lmx1b*-misexpressed embryo at E7.5. Dorsal view (A); view from the caudal side (B); transverse section stained with Hematoxylin-Eosin (C). Expansion of the tectum, torus semicircularis and rhombic lip are seen on the experimental side. (D-H) Morphology after *Wnt1* misexpression. E3.5 (48 hours after electroporation; D,E). E14.5 (13 days after electroporation; F-H). Dorsal view (D,F,G); view from the caudal side (E); horizontal section stained with Hematoxylin-Eosin (H). Extra folia (arrows on H) were formed in the cerebellum by *Wnt1* misexpression. Scale bars: 2 mm (F), 1 mm (B,G), 500 μm (C,E,H). cer, cerebellum; cont., control side; exp., experimental side; met, metencephalon; rl, rhombic lip; tec, tectum; ts, torus semicircularis.

We wondered whether expansion of the mesencephalon was caused by an increase of cell proliferation, and examined BrdU incorporation after 48 and 72 hours of electroporation of pMiw-Wnt1. Anti-BrdU staining revealed that BrdU incorporation was actually increased at the experimental side compared with the control at 48 hours after electroporation (Fig. 3). For the quantitative analysis, the BrdU-positive area between the corresponding site of the experimental and control side on the same section was compared as a pair. Six pairs from two embryos of 48 hours after electroporation showed that BrdU incorporation was significantly greater at the experimental side than at the control side (Table 1, $P<0.05$, Student's *t*-test). Difference in BrdU incorporation between the experimental and control side was not recognized 72 hours after electroporation (data not shown).

As *Wnt1* enhanced cell proliferation, it is of great interest if Wnt1 represses neuronal differentiation. So, we looked at effects on a neurogenesis marker, *Cash1*. Cash1 forces cells to get into differentiation phase from proliferation phase (Jasoni et al., 1994). *Wnt1* misexpression repressed *Cash1* expression (n=3/4) (see Fig. 6A) in the dorsal mesencephalon. Limx1b also exerted similar effects (n=3/3) (Fig. 4B).

Regulation of *Fgf8* by Lmx1b

As *Lmx1b* or *Wnt1* misexpression affected development of the

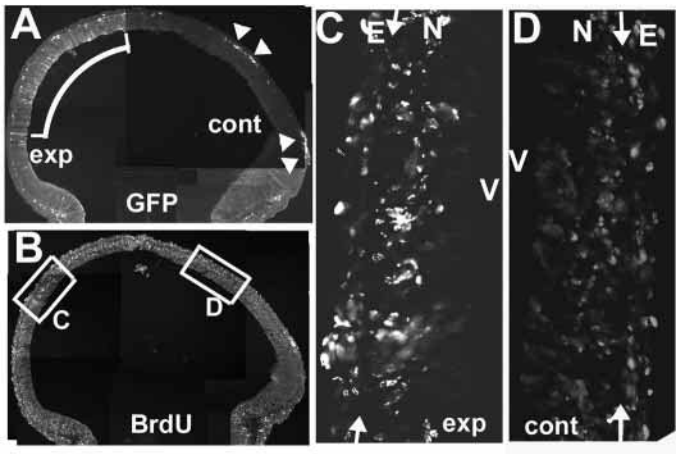

Fig. 3. BrdU incorporation after *Wnt1* misexpression. (A) GFP fluorescence micrograph to show misexpression site (marked by curved bar). Arrowheads indicate nonspecific fluorescence caused by blood cells. (B-D) Fluorescence micrographs for BrdU incorporation. Rectangles on low-power micrograph (B) indicate the area of C and D. The cryosections include surface ectoderm (arrows in C and D indicate the border between neuroepithelium and the surface ectoderm). V, ventricle; E, surface ectoderm; N, neuroepithelium; exp, experimental side; cont, control side.

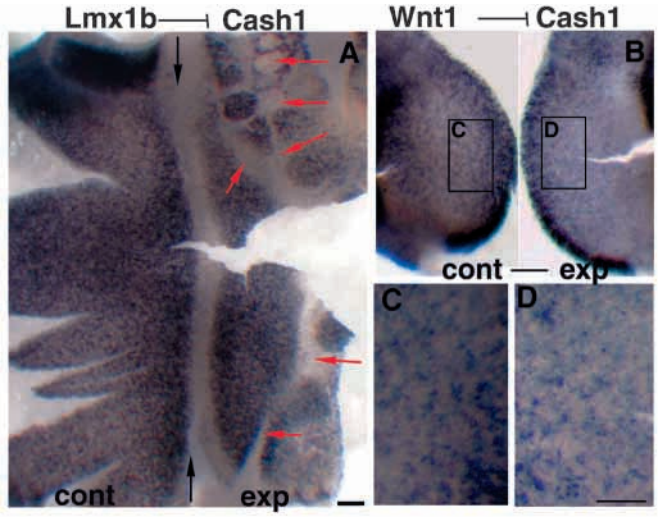

Fig. 4. Repression of *Cash1* by *Lmx1b* and *Wnt1* misexpression. (A) *Lmx1b* misexpression represses *Cash1* expression at 48 hours after electroporation. The right-hand side is the experimental side. Rostral is towards the top. Black arrows indicate dorsal midline. By 48 hours after electroporation, some regulation may have occurred, and repression sites are patchy (red arrows). (B) *Wnt1* misexpression represses *Cash1* expression at 24 hours after electroporation. Both panels are dorsal views of the mesencephalic region of embryos. (A) Flat mount; (B) dorsal view. (C,D) Higher magnification of the areas indicated in B. At 24 hours after electroporation, *Cash1* expression is repressed uniformly by Wnt1. Scale bars: 200 μm in A,B; 100 μm in C,D.

mesencephalon and metencephalon, we looked at effects on isthmus-related genes.

Effects of Lmx1b on *Fgf8* expression are not simple. *Fgf8* expression was repressed in *Lmx1b*-expressing cells (Fig. 5C). Repression of *Fgf8* by Lmx1b was already detectable at 12 hours after electroporation (n=4/4) (Fig. 5A-C). At 24 hours after electroporation, *Fgf8* was still repressed in the *Lmx1b*-expressing cells, but around them *Fgf8* expression was induced (n=3/7) (Fig. 5D-F). The results suggest that Lmx1b repressed *Fgf8* expression in a cell-autonomous manner, but induced Fgf8 expression in non cell-autonomous manner.

It has been reported that *Fgf8* is induced at the border of *Otx2* and *Gbx2* expression domain, overlapping with Gbx2 expression (Broccoli et al., 1999; Millet et al., 1999; Katahira et al., 2000; Li and Joyner, 2001; Ye et al., 2001). It has also been reported that Otx2 and Fgf8 repress each other's expression. Thus, a possibility remains that Otx2 is involved in cell-autonomous repression of *Fgf8* by Lmx1b, that is, Lmx1b at first induces *Otx2* expression then Otx2 represses *Fgf8* in turn. To check this possibility, we looked at effects of Lmx1b on *Otx2* expression. We then carried out *Otx2* misexpression, and looked at the time course of *Fgf8* repression by Otx2.

Table 1. Quantitative analysis of BrdU incorporation at 48 hours of pMiw-Wnt1 electroporation

Number of pairs	Area/10000 μm²		Difference
	Experimental	Control	
1	431.6	59.9	371.7
2	452.7	110.6	342.1
3	1764.5	189.7	1574.8
4	2191.4	388.5	1802.9
5	929.3	711.5	217.8
6	969.9	262.0	707.9
Mean±s.e.m.	1123.2±291.1	376.8±96.7	836.2±279.2

BrdU-positive areas from corresponding site of the experimental and control sides on the same section were extracted by the Image Analyzer (Aqua Cosmos, Hamamatsu Photonics) and compared as a pair. Six pairs from two embryos were analyzed. BrdU incorporation was significantly greater on the experimental side than on the control side (P<0.05, Student's t-test).

At 12 hours after *Lmx1b* misexpression, *Otx2* expression was induced ectopically in the metencephalon (n=3/3) (Fig. 6A-C′). *Otx2* was induced in the *Lmx1b*-expressing cells, suggesting that induction is cell-autonomous (Fig. 6B′,C′). At 24 hours after electroporation, ectopic *Otx2* expression became weak in the isthmic region, but strong in the caudal metencephalon (n=3/4) (Fig. 6D-F).

At 12 hours after electroporation of pMiw-Otx2, *Fgf8* expression was not affected (n=8/8) (Fig. 6G-I), which contrasts the result that repression of *Fgf8* expression by Lmx1b was detected by 12 hours after electroporation (Fig. 5C). These results indicate that Otx2 is not involved in cell-autonomous repression of *Fgf8* by Lmx1b.

Dominant-negative Lmx1b induced ectopic *Fgf8* expression in the mesencephalon

Lmx1b is a LIM-homeodomain protein, and is composed of two LIM domains, a homeodomain and a C-terminal transcription activation domain (Johnson et al., 1997). It has been suggested that LIM domain could work as dominant negative, and that deletion of LIM domain could work as constitutional activation of the target gene (Curtiss and Heiling, 1998). In case of Lmx1b, it was shown that deletion of LIM domain resulted in increase of transcription activity in

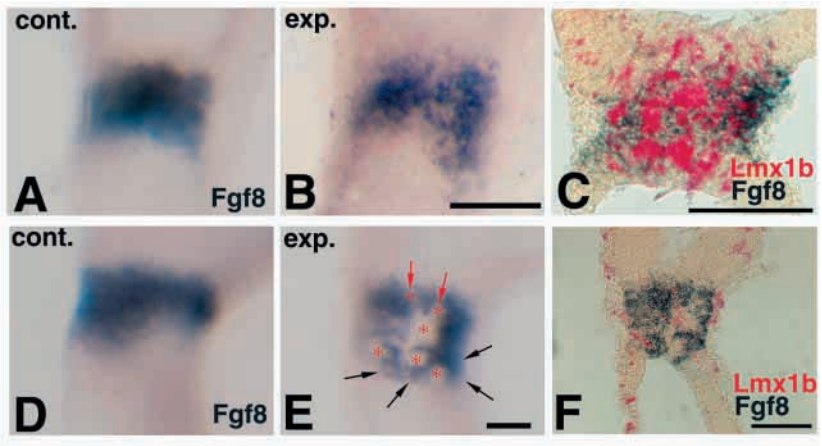

Fig. 5. Regulation of *Fgf8* by Lmx1b. (A-C) Cell-autonomous repression of *Fgf8* by Lmx1b. Whole-mount in situ hybridization for *Fgf8* (blue) at 12 hours after electroporation (A, control; B, experimental side). Section of the same embryo stained for *Fgf8* (blue) and *Lmx1b* (red) (C). Note that *Fgf8* expression was repressed in the *Lmx1b*-expressing cells (C). (D-F) Non cell-autonomous induction of *Fgf8* by Lmx1b. Whole-mount in situ hybridization for *Fgf8* (blue) at 24 hours after electroporation (D, control; E, experimental side). Section of the same specimen stained for *Fgf8* (blue) and *Lmx1b* (red) (F). *Fgf8* is still repressed in *Lmx1b*-misexpressing cells (asterisks indicated by red arrows, E), but around the *Lmx1b*-expressing cells (asterisks), Fgf8 is induced ectopically in the caudal metencephalon (black arrows in E). Scale bars: 100 μm. Views from the control side are printed in reverse for the comparison with the experimental side throughout the paper.

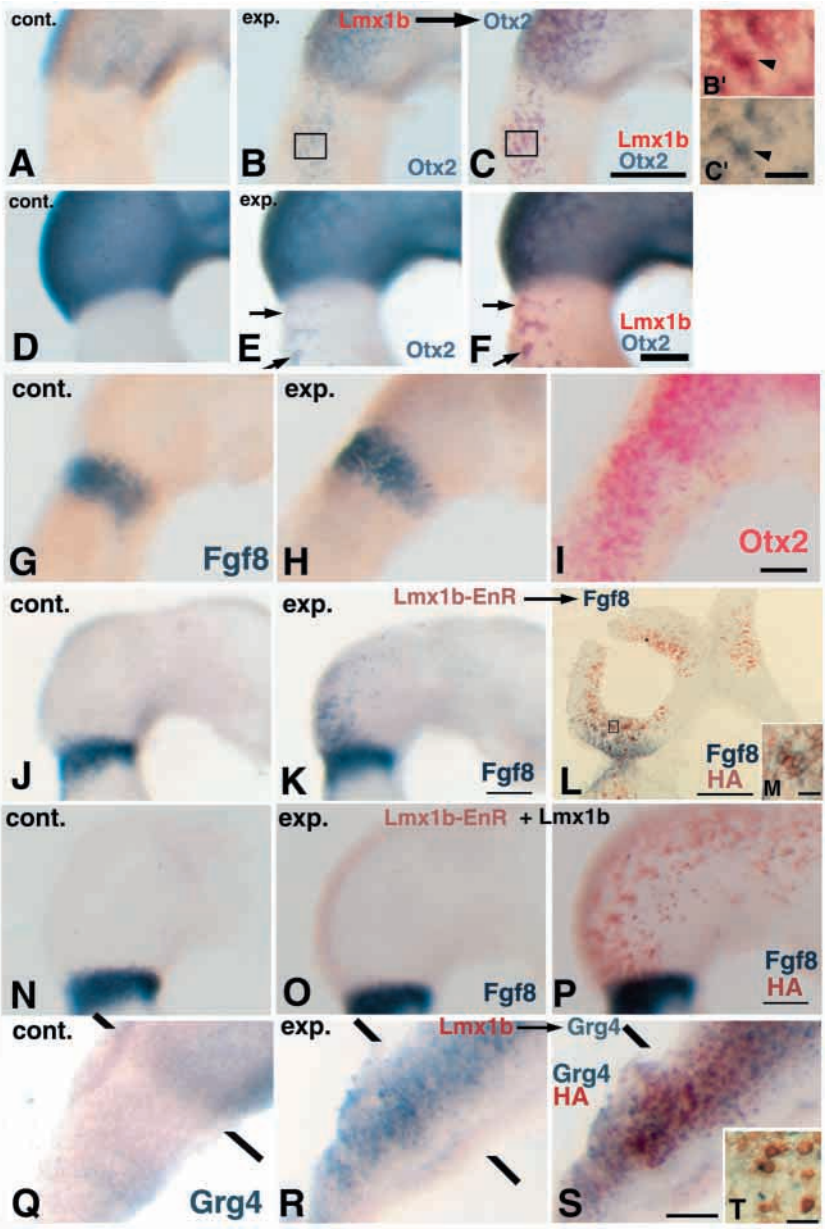

Fig. 6. Effects of *Lmx1b* misexpression on downstream gene expression. (A-F) Effects on *Otx2* expression. In situ hybridization for *Otx2* (blue) and *Lmx1b* (red) on the same embryo at 12 hours (A-C′) and 24 hours (D-F) after electroporation. (B′,C′) High-power magnification of boxed areas in B,C. *Otx2* is induced by *Lmx1b* misexpression, but only weakly induced in the isthmus at 24 hours after electroporation (E,F, arrows). (G-I) Effects of *Otx2* misexpression on *Fgf8* expression. In situ hybridization for *Fgf8* (blue) and *Otx2* (red) at 12 hours after *Otx2* misexpression. Repression of *Fgf8* is hardly observed at 12 hours after electroporation (H). (J-M) Effects of *Lmx1b-EnR* misexpression on *Fgf8* expression. In situ hybridization for *Fgf8* (J, control; K, experimental side). (L) Section of the specimen shown in J and K, in situ hybridization for *Fgf8* (blue) and immunohistochemical staining for HA tag (tagged to Lmx1b-EnR). (M) High power magnification of area in L. As Lmx1b is revealed by immunohistochemical staining, it is localized in the nucleus. *Fgf8* signal is localized in the cytoplasm. *Fgf8* is induced in *Lmx1b*-EnR misexpressing cells (M) in the caudal mesencephalon (K,L). (N-P) Effects of co-electroporation of *Lmx1b-EnR* and *Lmx1b* on *Fgf8* expression. In situ hybridization for *Fgf8* at 24 hours after electroporation (blue, N,O). (P) In situ hybridization for *Fgf8* (blue) and immunohistochemical staining for HA tag (tagged to Lmx1b-EnR). Ectopic expression of *Fgf8* in the caudal mesencephalon is canceled by co-electroporation with wild-type *Lmx1b*. (Q-T) Effects of L*mx1b* misexpression on *Grg4* expression. In situ hybridization for *Grg4* (blue) (Q,R), and immunostaining for HA (tagged to Lmx1b, brown) (S) at 6 hours after electroporation of pMiw-Lmx1b. *Grg4* is induced in the metencephalon on the experimental side (R,S). (T) High-power magnification of the metencephalic region to show that Grg4 is induced in the *Lmx1b*-misexpressed cells. (A,D,G,J,N,Q) Views from the control side. (B,C,E,F,H,I,K-M,O,P,R-T) Views from the experimental side. Scale bars: 250 μm in F; 200 μm in C,I,K,L,P; 100 μm in S; 25 μm in C′; 10 μm in M,T.

insulin enhancer in vitro (German and Wang, 1994; Johnson et al., 1997).

As Lmx1b repressed *Fgf8* expression in cell-autonomous manner, we wondered if Lmx1b functions as transcriptional repressor or activator. To answer this question, we misexpressed N-terminal deletion construct of *Lmx1b* (*Lmx1b-C*), in which LIM domain is not contained. Lmx1b-C misexpression exerted weak but similar effects as Lmx1b misexpression. In the metencephalon, Lmx1b-C induced *Fgf8* expression around the cells where *Lmx1b-C* was misexpressed at 24 hours after electroporation (*n*=8/13).

We tried misexpression of LIM domain in order to repress *Lmx1b* function, but it did not work. So, we constructed an expression vector that encodes the fusion protein of Lmx1b and En2 repressor domain. *Lmx1b-EnR* misexpression induced ectopic expression of *Fgf8* in the caudal mesencephalon in a cell-autonomous manner (*n*=7/7) (Fig. 6J-M). Co-transfection of wild type *Lmx1b* and *Lmx1b-EnR* canceled the effect of Lmx1b-EnR (*n*=8/8) (Fig. 6N-P), which indicates that *Lmx1b-EnR* specifically repressed function of Lmx1b. The results suggest that Lmx1b acts as a transcriptional activator in the mes-metencephalic region.

Candidate repressor of Fgf8

As Lmx1b acted as a transcriptional activator, some repressor(s) should intervene in repression of *Fgf8* by Lmx1b. It was indicated that Grg4 interacts with the octapeptide domain of Pax2/5 (Eberhand et al., 2000) to convert it to transcriptional repressor. *Grg4* is expressed in the mesencephalon but not in the isthmus in normal development, (Fig. 6Q) (Koop et al., 1996; Sugiyama et al., 2000; Ye et al., 2001), and *Grg4* misexpression resulted in repression of *Fgf8* expression in the isthmus (Sugiyama et al., 2000). Therefore, we examined the effects of Lmx1b on *Grg4* expression. At 6 hours after electroporation of pMiw-Lmx1b, *Grg4* expression was induced in the metencephalon, (*n*=5/7) (Fig. 6R,S). As repression of *Fgf8* by Lmx1b was not observed before 6 hours after electroporation (*n*=4/4) and induction of *Grg4* occurred before *Fgf8* repression, it is plausible that Lmx1b first induced *Grg4* and then Grg4 repressed *Fgf8*.

Wnt1 misexpression affects *Fgf8* expression

As *Lmx1b* induced *Fgf8* non cell-autonomously, secreted molecules may be involved in this process. Wnt1 is a secreted molecule expressed in an overlapping manner with *Lmx1b*. So, we suspected that Lmx1b induced *Fgf8* via *Wnt1* induction. By 9 hours after electroporation of *Lmx1b*, *Wnt1* was induced in cell-autonomous manner (*n*=1/3). By 24 hours after electroporation, *Wnt1* was induced broadly (*n*=6/6) (Fig. 7A-E). Then, we examined if *Fgf8* could be induced by Wnt1 to assess our idea. As expected, *Fgf8* was induced by Wnt1 in the metencephalic region by 12 hours after electroporation (*n*=2/4). At 24 hours after electroporation of pMiw-Wnt1, *Fgf8* expression was expanded in the metencephalic region (*n*=6/6) (Fig. 7G). But *Fgf8* was not induced in the mesencephalon and in the caudal metencephalon, though misexpression was seen from the mesencephalon to the metencephalon (Fig. 7G,H).

Next, we checked effects of Wnt1 on *Lmx1b* expression. *Lmx1b* expression was not affected by 24 hours after pMiw-Wnt1 electroporation. (*n*=6/6) (Fig. 7I-K). Wnt1 did not affect *Otx2* (*n*=7/7) or *Gbx2* (*n*=7/7) expression at 24 hours after electroporation (Fig. 7L-Q). As *Wnt1* was induced by Lmx1b, *Lmx1b* may occupy higher hierarchical position in gene expression cascade in the isthmic region.

Further analysis in gene expression cascade among *Lmx1b, Otx2*, *Gbx2* and *Fgf8*

We have shown that *Lmx1b* may be put at the higher

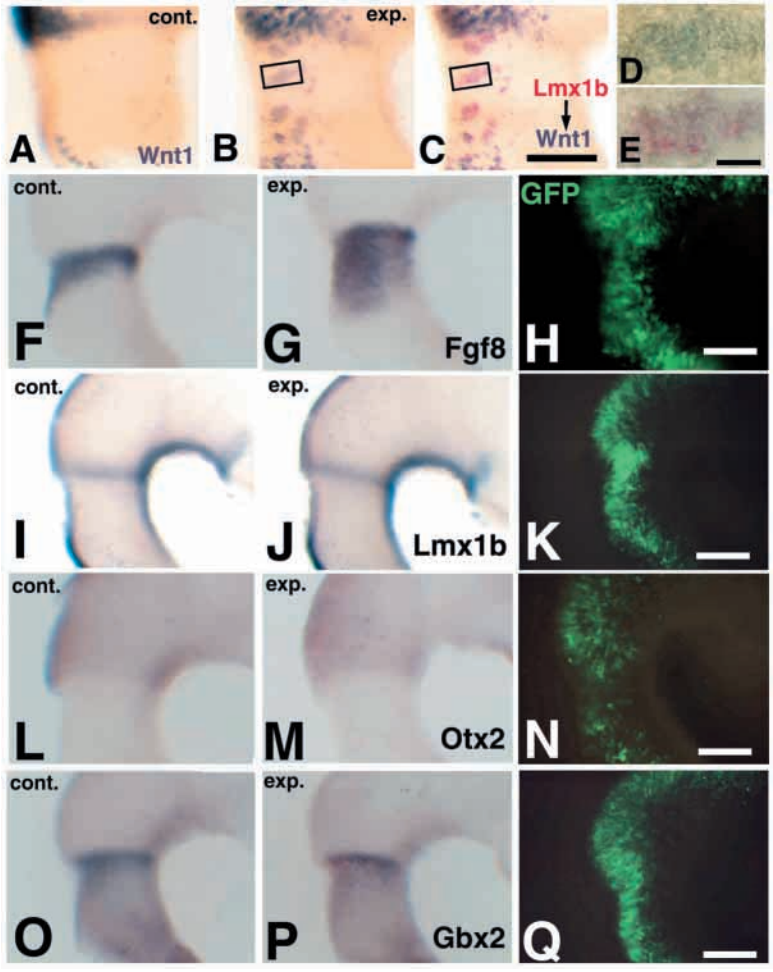

Fig. 7. Effects of *Lmx1b* and*Wnt1* misexpression on isthmus-related genes. (A-E) *Wnt1* induction by *Lmx1b* misexpression. In situ hybridization for *Wnt1* (blue; A,B,D) and *Lmx1b* (red; C,E) on the same embryo 24 hours after electroporation. (A) View from the control side; (B,C) view from the experimental side. (D,E) High-power magnifications of boxed areas in B,C, respectively. *Wnt1* is expressed in the *Lmx1b*-expressing cells (D,E). (F-Q) Effects of *Wnt1* misexpression on *Fgf8*, *Otx2*, *Gbx2* and *Lmx1b*. In situ hybridization for *Fgf8* (F,G), *Lmx1b* (I,J), *Otx2* (L,M) and *Gbx2* (O,P). (F,I,L,O) View from the control sides. (G,J,M,P) View from the experimental side. (H,K,N,Q) Fluorescence micrograph of GFP to show transfection efficiency. *Fgf8* expression expanded caudally by *Wnt1* misexpression (G). *Lmx1b*, *Otx2* and *Gbx2* expression is not affected (J,M,P). Scale bars: 500 μm in H,K,N,Q; 200 μm in C; 50 μm in E.

hierarchical position in gene expression cascade in the isthmus and may play important roles in mes/mesencephalic development, so we further analyzed their relationship. We have already shown that Lmx1b could induce *Otx2*. Then we examined if Otx2 could induce *Lmx1b*. At 24 hours after electroporation of pMiw-Otx2, *Lmx1b* expression was induced in the mesencephalon and the metencephalon (*n*=4/6) (Fig. 8A-C). The result indicates that Otx2 and Lmx1b could induce each other's expression.

Next, we looked at the effects of Fgf8 on *Lmx1b* expression. *Lmx1b* was broadly induced in the diencephalon and mesencephalon by *Fgf8b* misexpression at 24 hours after electroporation (*n*=4/4) (Fig. 8D-F). However, by 36 hours after electroporation, *Lmx1b* expression disappeared from most part of the mesencephalon and ring-like expression in the diencephalon remained, although misexpression was seen broadly when checked by GFP, (*n*=4/4) (Fig. 8G,H). Moreover, endogenous *Lmx1b* expression in the isthmus was lost (Fig. 8H).

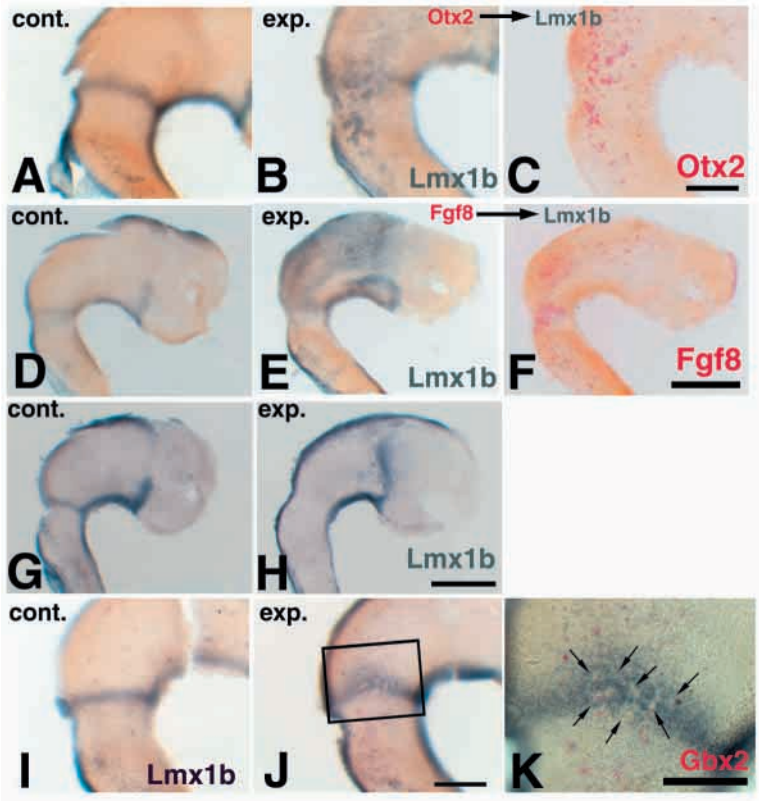

Fig. 8. Effects of Otx2, Fgf8 and Gbx2 on *Lmx1b* expression. (A-C) In situ hybridization for *Lmx1b* (blue) and *Otx2* (red) on the same embryo at 24 hours after electroporation of pMiw-Otx2. *Lmx1b* is induced by *Otx2* misexpression in the metencephalon (B). (D-F) In situ hybridization for *Lmx1b* (blue) and *Fgf8* (red) on the same embryo at 24 hours after electroporation of pMiw-Fgf8b. *Lmx1b* is induced by *Fgf8b* misexpression in the diencephalon and mesencephalon broadly (E). (G,H) In situ hybridization for *Lmx1b* (blue) on the embryo at 36 hours after electroporation of pMiw-Fgf8b. (H) *Lmx1b* expression appears as a half ring in the diencephalon. In addition, endogenous *Lmx1b* expression in the isthmus is also disappeared (H). (I-K) In situ hybridization for *Lmx1b* (blue) and *Gbx2* (red) on the same embryo at 24 hours after electroporation of pMiw-Gbx2. (K) High-power magnification of J. *Lmx1b* was repressed by *Gbx2* misexpression (indicated by arrows on K). (A,D,G,I) Views from the control side. (B,C,E,F,H,J,K) Views from the experimental sides. Scale bars: 500 μm in F,H; 250 μm in C,J; 100 μm in K.

Fgf8b misexpression induced *Gbx2* and *Irx2* expression widely in the mesencephalon [see figure 7D,E by Sato et al. (Sato et al., 2001)], and changed the fate of the mesencephalic alar plate to differentiate into the cerebellum (Sato et al., 2001). As Fgf8 induces *Gbx2* expression, we wondered if *Lmx1b* was repressed by Gbx2. To examine this possibility, we misexpressed *Gbx2* and looked at *Lmx1b* expression. *Gbx2* repressed *Lmx1b* expression at 24 hours after electroporation (*n*=4/4) (Fig. 8I-K).

DISCUSSION

In the present study, we have shown that: (1) expression domains of *Lmx1b* and *Wnt1* were gradually segregated at the isthmus from that of *Fgf8*; (2) both *Lmx1b* and *Wnt1* misexpression resulted in enlargement of the tectum and the cerebellum; (3) *Fgf8* expression was repressed in *Lmx1b* misexpressing cells, but *Fgf8* was induced around the *Lmx1b* misexpressing cells; (4) Lmx1b induced *Wnt1*, *Otx2* and *Grg4* cell-autonomously; (5) Wnt1 induced *Fgf8* expression non cell-autonomously; and (6) *Lmx1b* is induced by Otx2 and Fgf8, but repressed by Gbx2. The possible role of Lmx1b in the formation and maintenance of isthmus organizer is discussed below.

Role of Lmx1b and Wnt1 in the isthmus organizer formation

Transplantation experiments showed that isthmic region has the organizing activity for the tectum and cerebellum. As Fgf8 beads mimic the isthmus organizing activity, and misexpression of Fgf8b changed the fate of the mesencephalic alar plate to differentiate into the cerebellum, it has been accepted that Fgf8 is the most important organizing molecule (Crossley et al., 1996; Liu et al., 1999; Martinez et al., 1999; Shamim et al., 1999; Sato et al., 2001). En1/2, Pax2/5 and Fgf8 could induce each other's expression, and this positive feedback loop of En1/2, Pax2/5 and Fgf8 may play an important role for the maintenance of the organizing activity. If one of these molecules is misexpressed in the diencephalon, this feedback loop is turned on, and the ectopic tectum is induced in the diencephalon.

Lmx1b and *Wnt1* are expressed in the whole mesencephalon at first, and localized to the isthmus. As *Wnt1* knock-out mice show deletion in the midbrain and hindbrain, it was suggested that Wnt1 is necessary for mid-hindbrain development (McMahon and Bradley, 1990; Thomas and Capecchi, 1991; McMahon et al., 1992). But misexpression of *Wnt1* by retrovirus vector or by transplanting the Wnt1-producing cells did not exert significant effects on mid-hindbrain development (Sugiyama et al., 1998; Adams et al., 2000), so its role in this system is still obscure. Recently, *Lmx1b* was misexpressed by retrovirus vector, and it was suggested that *Lmx1b* should be given higher hierarchical position than *Wnt1* in the gene expression cascade in the isthmus (Adams et al., 2000). In the present study, we have shown by misexpression by in ovo electroporation that *Fgf8*

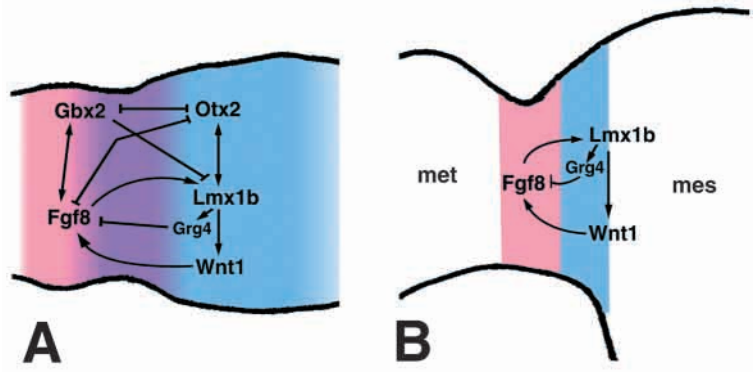

Fig. 9. Role of Lmx1b and Wnt1 in isthmus organizing activity. At 10-somite stage, *Lmx1b* and *Wnt1* (blue) are expressed in the mesencephalon and metencephalon (A). Their expression overlaps with *Fgf8* (red) expression around the isthmic region. Lmx1b represses *Fgf8* cell-autonomously; however, it induced *Fgf8* expression non cell-autonomously in adjacent cells. In cell-autonomous repression of *Fgf8* by Lmx1b, Grg4 may intervene. In non cell-autonomous induction of *Fgf8* by Lmx1b, Wnt1 may be involved. Fgf8 could induce *Lmx1b*. Gbx2 and Fgf8 induces each other's expression, and Gbx2 repressed *Lmx1b* expression. Otx2 and Gbx2 repress each other's expression. As a result of this complicated gene expression cascade, *Fgf8* expression may be set and kept in the isthmic region just rostral to the Lmx1b and Wnt1 expression ring by E2.5 (B).

expression was repressed in *Lmx1b* misexpressing cells, but *Fgf8* expression was induced around *Lmx1b* misexpressing cells. This complicated phenomenon may be explained as follows. First, *Wnt1* may be induced by Lmx1b in cell-autonomous manner, then Wnt1 may induce *Fgf8* expression in turn. As Wnt1 is a secreted molecule, Wnt1 may be involved in non cell-autonomous induction of *Fgf8* by Lmx1b.

Several molecules are abutting at the mid-hindbrain boundary (Fig. 9). At the midbrain side, *Otx2*, *Lmx1b* and *Wnt1* are expressed, and *Gbx2*, *Fgf8* and *Irx2* are expressed at the hindbrain side. Otx2 and Gbx2, Fgf8 and Otx2, and Lmx1b and Gbx2, repress each other's expression. However, Lmx1b cell-autonomously represses *Fgf8*, but induces *Fgf8* in the neighboring cells. Otx2 could not induce *Fgf8* expression. By these complicated gene regulation mechanisms gene expression pattern in the isthmic region may be set, in a sense automatically, once initial switch of some of them is turned on. *Fgf8b* misexpression may be one of such cases of autoregulation. After *Fgf8b* misexpression, Lmx1b was induced widely in the mesencephalon at first, but later *Lmx1b* expression was restricted in the diencephalic region just as ring, which reminds us of ring like expression in the isthmus in normal embryos. This self regulation may be explained if we consider that *Gbx2* is also induced by Fgf8b (Sato et al., 2001). As Gbx2 represses *Lmx1b* expression (see Fig. 7I-K), *Lmx1b* expression, which was induced broadly in the mesencephalon at first, may be repressed by Gbx2. *Lmx1b* expression may remain just outside of the Gbx2 area to result in ring-like expression in the diencephalic region. Another example is that Fgf8 was induced when R1 and midbrain was juxtaposed (Hidalgo-Sanchez et al., 1999). This phenomenon may be explained by that *Otx2* and *Lmx1b* are expressed in the mesencephalic region, and that *Wnt1* may be induced by Lmx1b. As Wnt1 is a secreted molecule, it may induce *Fgf8* expression in non cell-autonomous manner, that is, in the R1 region. The intimate relationship of these molecules may participate in setting the site of organizer, and mid-hindbrain boundary.

In the isthmic region many molecules are expressed, and they are in the complicated network of regulation. Fgf8, Pax2/5 and En1 are in the positive feedback loop for their expression. Pax2 expression covers whole the mesencephalon and comes to be localized in the isthmic region (Okafuji et al., 1999). As it could induce *Fgf8* in the diencephalon, Pax2 has also been suggested to be involved in *Fgf8* induction (Okafuji et al., 1999; Ye et al., 2001). It was further shown that in *Pax2*$^{-/-}$ mice *Fgf8* expression in the isthmus was abolished though its expression in the cardiac mesoderm was not affected (Ye et al., 2001).

In normal development, *Otx2* and *Gbx2* are expressed from very early stage of development. At first their expression domains are overlapping, but are completely segregated around stage 10 in chick embryos. It was shown that Otx2 and Gbx2 repress each other's expression so that their expression domains become segregated. By the repressive interaction between Otx2 and Gbx2, mid-hindbrain boundary may be set. Independently, *Pax2* expression may be induced by the vertical signal, and *Fgf8* may be induced in the isthmic region. Considering appearance of *Lmx1b* expression and its induction by Otx2, *Lmx1b* may be induced by Otx2 in the midbrain region, though there is a possibility that vertical signal contribute to induction of *Lmx1b*. *Wnt1* may be induced in turn. However, *Fgf8* expression was not affected at first in *Wnt1*$^{-/-}$ mice, but was later disrupted (Lee et al., 1997). This result together with the misexpression experiments including the present study that Lmx1b or Wnt1 did not exert severe morphological effects, indicates that Lmx1-Wnt1 system may work to maintain *Fgf8* expression rather than initiation of its expression. *Fgf8* expression may be kept just caudal to the *Wnt1* and *Lmx1b* expression ring (Fig. 9).

Growth accelerating activity of Wnt1

Both *Lmx1b* and *Wnt1* misexpression caused enlargement of cerebellum or rhombic lip and the tectum. Extra folia were developed in the cerebellum. As Lmx1b induces *Wnt1* expression, both Lmx1 and Wnt1 may have exerted similar effect. It has been reported that *Wnt1* transgenic mice show overgrowth of neural tube (Dickinson et al., 1994). *Wnt1* was misexpressed under the control of *Hoxb4* enhancer, which resulted in dramatic increase in the number of mitosis in the ventricular layer and expansion of it. Very recently, Megason and McMahon (Megason and McMahon, 2002) reported in a very sophisticated manner that Wnt protein is distributed in a dorsal to ventral gradient in the spinal cord. They suggested that Wnt-β-catenin/TCF signaling pathway positively regulates cell cycle progression and negatively regulates cell cycle exit in the spinal cord through transcriptional regulation of cyclin D1 and cyclin D2. In the present study, *Cash1* was repressed by both Lmx1b and Wnt1, which may indicates that ventricular cells in the tectum are also prevented from getting into differentiation phase. In the tectum anlagen, more cells incorporated BrdU at the *Wnt1*-transfection site than at the control side at 48 hours after electroporation (see Fig. 3).

Difference in BrdU incorporation was not discerned at 72 hours after electroporation. The results indicate that *Wnt1* actually enhanced cell proliferation, but the effect on BrdU incorporation was transient so that the size difference between the Wnt1-transfected and the control tecta may have been subtle.

We thank Dr T. Reh for *Cash1* probe and Dr I. Araki for *Wnt1* cDNA. This work was supported by the Ministry of Education, Culture, and Sports, Science and Technology, Japan, and by the Mitsubishi Foundation.

REFERENCES

Adams, K. A., Maida, J. M., Golden, J. A. and Riddle, R. D. (2000). The transcription factor Lmx1b maintains Wnt1 expression within the isthmic organizer. *Development* **127**, 1857-1867.

Alvarado-Mallart, R.-M. (1993). Fate and potentialities of the avian mesencephalic/metencephalic neuroepithelium. *J. Neurobiol.* **24**, 1341-1355.

Araki, I. and Nakamura, H. (1999). *Engrailed* defines the position of dorsal di-mesencephalic boundary by repressing diencephalic fate. *Development* **126**, 5127-5135.

Bally-Cuif, L., Cholley, B. and Wassef, M. (1995). Involvement of Wnt-1 in the formation of the mes/metencephalic boundary. *Mech. Dev.* **53**, 23-34.

Broccoli, V., Boncinelli, E. and Wurst, W. (1999). The caudal limit of *Otx2* expression positions the isthmic organizer. *Nature* **401**, 164-168.

Crossley, P. H., Martinez, S. and Martin, G. R. (1996). Midbrain development induced by FGF8 in the chick embryo. *Nature* **380**, 66-68.

Curtiss, J. and Heilig, J. S. (1998). DeLIMiting development. *BioEssays* **20**, 58-69.

Dickinson, M. E., Krumlauf, R. and McMahon, A. P. (1994). Evidence for a mitogenic effect of Wnt-1 in the developing mammalian central nervous system. *Development* **120**, 1453-1471.

Eberhard, D., Jimenez, G., Heavey, B. and Busslinger, M. (2000). Transcriptional repressor by Pax5 (BSAP) through interaction with corepressor of the Groucho family. *EMBO J.* **19**, 2292-2303.

Funahashi, J.-i., Okafuji, T., Ohuchi, H., Noji, S., Tanaka, H. and Nakamura, H. (1999). Role of Pax-5 in the regulation of a mid-hindbrain organizer's activity. *Dev. Growth. Differ.* **41**, 59-72.

German, M. S. and Wang, J. (1994). The insulin gene contains multiple transcriptional elements that respond to glucose. *Mol. Cell. Biol.* **14**, 4067-4075.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.

Hidalgo-Sanchez, M., Simeone, A. and Alvarado-Mallart, R.-M. (1999). *Fgf8* and *Gbx2* induction concomitant with *Otx2* repression is correlated with midbrain-hindbrain fate of caudal prosencephalon. *Development* **126**, 3191-3203.

Irving, C. and Mason, I. (1999). Regeneration of isthmic tissue is the result of a specific and direct interaction between rhombomere 1 and midbrain. *Development*, **126**, 3981-3989.

Ishii, Y., Fukuda, K., Saiga, H., Matsushita, S. and Yasugi, S. (1997). Early specification of intestinal epithelium in the chicken embryo: a study on the localization and regulation of CdxA expression. *Dev. Growth. Differ.* **39**, 643-653.

Jasoni, C. L., Walker, M. B., Morris, M. D. and Reh, T. A. (1994). A chicken achaete-scute homolog (CASH-1) is expressed in a temporally and spatially discrete manner in the developing nervous system. *Development* **120**, 769-783.

Johnson, J. D., Zhang, W., Rudnick, A., Rutter, W. and German, M. (1997). Transcriptional synergy between LIM-homeodomain proteins and basic helix-loop-helix proteins: the LIM2 domain determines specificity. *Mol. Cell. Biol.* **17**, 3488-3496.

Katahira, T., Sato, T., Sugiyama, S., Okafuji, T., Araki, I., Funahashi, J.-I. and Nakamura, H. (2000). Interaction between Otx2 and Gbx2 defines the organizing center for the optic tectum. *Mech. Dev.* **91**, 43-52.

Koop, K. E., MacDonald, L. M. and Lobe, C. G. (1996). Transcripts of Grg4, a murine groucho-related gene, are detected in adjacent tissues to other murine neurogenic gene homologues during embryonic development. *Mech. Dev.* **59**, 73-87.

Lee, S. M., Danielian, P. S., Fritzsch, B. and McMahon, A. P. (1997). Evidence that FGF8 signalling from the midbrain-hindbrain junction regulates growth and polarity in the developing midbrain. *Development* **124**, 959-969.

Li, J. Y. and Joyner, A. L. (2001). Otx2 and Gbx2 are required for refinement and not induction of mid-hindbrain gene expression. *Development* **128**, 4979-4991.

Liu, A., Losos, K. and Joyner, A. L. (1999). FGF8 can activate Gbx2 and transform regions of the rostral mouse brain into a hindbrain fate. *Development* **126**, 4827-4838.

Marin, F. and Puelles, L. (1994). Patterning of the embryonic avian midbrain after experimental inversions: a polarizing activity from the isthmus. *Dev. Biol.* **163**, 19-37.

Martinez, S., Wassef, M. and Alvarado-Mallart, R. M. (1991). Induction of a mesencephalic phenotype in the 2-day-old chick prosencephalon is preceded by the early expression of the homeobox gene *en*. *Neuron* **6**, 971-981.

Martinez, S., Marin, F., Nieto, M. A. and Puelles, L. (1995). Induction of ectopic engrailed expression and fate change in avian rhombomeres: intersegmental boundaries as barriers. *Mech. Dev.* **51**, 289-303.

Martinez, S., Crossley, P. H., Cobos, I., Rubenstein, J. L. R. and Martin, G. R. (1999). FGF8 induces formation of an ectopic isthmic organizer and isthmocerebellar development via a repressive effect on *Otx2* expression. *Development* **126**, 1189-1200.

Matsunaga, E., Araki, I. and Nakamura, H. (2000). *Pax6* defines the di-mesencephalic boundary by repressing *En1* and *Pax2*. *Development* **127**, 2357-2365.

Matsunaga, E., Araki, I. and Nakamura, H. (2001). Role of *Pax3/7* in the tectum regionalizartion. *Development* **128**, 4069-4077.

McMahon, A. P. and Bradley, A. (1990). The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell* **62**, 1073-1085.

McMahon, A. P., Joyer, A., Bradley, A. and McMahon, J. A. (1992). The midbrain-hindbrain phenotype of *Wnt1-/Wnt1-* mice results from stepwise deletion of *engrailed*-expressing cells by 9.5 days postcoitum. *Cell* **69**, 581-595.

Megason, S. G. and McMahon, A. P. (2002). A mitogen gradient of dorsal midline Wnts organize growth in the CNS. *Development* **129**, 2087-2098.

Millet, S., Campbell, K., Epstein, D. J., Losos, K., Harris, E. and Joyner, A. L. (1999). A role for *Gbx2* in repression of *Otx2* and positioning the mid/hindbrain organizer. *Nature* **401**, 161-164.

Nakamura, H. and Funahashi, J.-i. (2001). Introduction of DNA into chick embryos by in ovo electroporation. *Methods* **24**, 43-48.

Okafuji, T., Funahashi, J. and Nakamura, H. (1999). Roles of Pax-2 in initiation of the chick tectal development. *Dev. Brain Res.* **116**, 41-49.

Sato, T., Araki, I. and Nakamura, H. (2001). Inductive signal and tissue responsiveness to define the tectum and the cerebellum, *Development* **128**, 2461-2469.

Shamim, H., Mahmood, R., Logan, C., Doherty, P., Lumsden, A. and Mason, I. (1999). Sequential roles for Fgf4, En1 and Fgf8 in specification and regionalisation of the midbrain. *Development* **126**, 945-959.

Suemori, H., Kadokawa, Y., Goto, K., Araki, I., Kondoh, H. and Nakatsuji, N. (1990). A mouse embryonic stem cell line showing pluripotency of differentiation in early embryos and ubiquitous beta-galactosidase expression. *Cell. Differ. Dev.* **29**, 181-186.

Sugiyama, S., Funahashi, J., Kitajewski, J. and Nakamura, H. (1998). Crossregulation between En-2 and Wnt-1 in chick tectal development. *Dev. Growth. Differ.* **40**, 157-166.

Sugiyama, S., Funahashi, J.-i. and Nakamura, H. (2000). Antagonizing activity of chick Grg4 against tectum-organizing activity. *Dev. Biol.* **221**, 168-180.

Thomas, K. R. and Capecchi, M. R. (1990). Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature* **346**, 847-850.

Wakamatsu, Y., Watanabe, Y., Nakamura, H. and Kondoh, H. (1997). Regulation of the neural crest cell fate by N-myc: promotion of ventral migration and neuronal differentiation. *Development* **124**, 1953-1962.

Ye, W., Bouchard, M., Stone, D., Liu, A., Vella, F., Lee, J., Nakamura, H., Ang, S.-L., Busslinger, M. and Rosenthal, A. (2001). Distinct regulators control the expression of the mid-hindbrain organizer signal FGF8. *Nat. Neurosci.* **4**, 1175-1181.

Yuan, S. and Schoenwolf, G. C. (1999). The spatial and temporal pattern of C-Lmx1 expression in the neuroectoderm during chick neurulation. *Mech. Dev.* **88**, 243-247.