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Fgfr1-dependent boundary cells between developing mid- and hindbrain

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Abstract

Signaling molecules regulating development of the midbrain and anterior hindbrain are expressed in distinct bands of cells around the midbrain–hindbrain boundary. Very little is known about the mechanisms responsible for the coherence of this signaling center. One of the fibroblast growth factor (FGF) receptors, *Fgfr1*, is required for establishment of a straight border between developing mid- and hindbrain. Here we show that the cells close to the border have unique features. Unlike the cells further away, these cells express *Fgfr1* but not the other FGF receptors. The cells next to the midbrain–hindbrain boundary express distinct cell cycle regulators and proliferate less rapidly than the surrounding cells. In *Fgfr1* mutants, these cells fail to form a coherent band at the boundary. The slowly proliferating boundary cells are necessary for development of the characteristic isthmic constriction. They may also contribute to compartmentalization of this brain region.

Keywords: FGF; Isthmic organizer; Cerebellum; Midbrain; Rhombomere; Development; Proliferation; Cyclin; Cell adhesion

Introduction

Planar signaling within the neural tube plays important role in the regionalization of the vertebrate brain. One source of such signals is the isthmic organizer (IsO), which is located around the border between the developing midbrain and rhombomere 1 of the hindbrain (referred to as midbrain–hindbrain boundary in the following; Bally-Cuif et al., 1992; Martinez et al., 1991; Nakamura, 1990).

Signaling molecules secreted by the IsO include fibroblast growth factor (FGF) family members *Fgf8*, *Fgf17*, and *Fgf18*, as well as *Wnt1* (for reviews, see Echevarria et al., 2003; Liu and Joyner, 2001; Nakamura, 2001; Wurst and Bally-Cuif, 2001). Initially, *Fgf8* and *Wnt1* are expressed in broad regions of rhombomere 1 and midbrain, respectively. However, at E9.5 expression of *Fgf8* and *Wnt1* is sharpened and their expressions abut at the boundary between midbrain and hindbrain. Both of these signaling molecules are essential for the early development of the midbrain–hindbrain region (Chi et al., 2003; McMahon and Bradley, 1990; Meyers et al., 1998; Reifers et al., 1998). In addition, ectopic application of FGF8 can alter the regional identity of diencephalon and posterior hindbrain, thus mimicking the IsO activity (Crossley et al., 1996; Irving and Mason, 2000; Martinez et al., 1999). FGF signaling from the IsO is important throughout development of the mid- and hindbrain region and at later stages *Fgf8* and *Fgf17* appear to have to some extent redundant functions (Xu et al., 2000).

In contrast to the large number of genes known to be important for the IsO activity, relatively little is known about the cellular properties of the midbrain–hindbrain boundary region and effects of IsO signals on them. For example, it is still controversial whether the early midbrain and rhombomere 1 of the hindbrain can be regarded as

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cell-lineage-restricted compartments or whether there is movement of cells across the border between them (Bally-Cuif et al., 1995; Jungbluth et al., 2001; Millet et al., 1996; Rhinn et al., 1999; Trokovic et al., 2003). It has been shown that IsO can stimulate cellular proliferation and suppress neuronal differentiation in a relatively broad region of midbrain and rhombomere 1 (Lee et al., 1997; Li et al., 2002; Xu et al., 2000). In contrast, cells next to the midbrain–hindbrain border have distinct shape and create a morphological constriction at the boundary. However, the characteristics and significance of these cells are poorly understood.

We have previously shown that one of the FGF receptors, FGFR1, is required for a sustained response to the signals from the IsO. In addition to regulating gene expression, FGFR1 may also be important for cell adhesive characteristics at the boundary. In the *Fgfr1* mutants, a straight border between the midbrain and hindbrain is not seen, but cells with midbrain and rhombomere 1 characteristics appear to mix with each other (Trokovic et al., 2003). However, phenotype of the embryos carrying midbrain and rhombomere-1-specific inactivation of the *Fgfr1* gene is less severe than that of the corresponding *Fgf8* mutants, in which the cells of the midbrain and anterior hindbrain die apoptotically at an early stage (Chi et al., 2003).

We show here that in addition to FGFR1 also other FGF receptors, FGFR2 and FGFR3, are potentially involved in transduction of the signals from the IsO. Interestingly however, the cells close to the midbrain–hindbrain boundary appear to express only *Fgfr1* and are dependent on *Fgfr1* for expression of target genes. These previously uncharacterized cells in the most posterior midbrain and most anterior rhombomere 1 express distinct set of cell cycle regulators and proliferate less rapidly that the cells further away from the boundary. Our results suggest that these cells are important for stabilization and coherence of the midbrain–hindbrain boundary.

Materials and methods

Generation and genotyping of mice

En1-Cre and $Fgfr1^{Flox}$ alleles have been described previously (Kimmel et al., 2000; Trokovic et al., 2003). Mutant embryos were generated by crossing *En1-Cre/+*; $Fgfr1^{Flox/+}$ males with $Fgfr1^{Flox/Flox}$ females in outbred (129sv/ICR) background. The generation of $En1^{+/Wnt1}$ mice has been reported previously (Panhuysen et al., 2004). Noon of the day of a vaginal plug was considered as E0.5 in the timing of embryos. More exact embryonic staging was estimated by counting the somites. For genotyping of the mice and embryos, see Trokovic et al. (2003). All the experiments were approved by the committee of experimental animal research of the University of Helsinki.

Histology

For paraffin sections, embryos were fixed overnight in 4% PFA at +4°C, dehydrated, embedded in paraffin and sectioned sagittally at 5 μm. In all cases, sagittal sections around the midline were collected.

For semithin sections, embryos were fixed overnight at room temperature in 5% glutaraldehyde (EM grade, Distillation Purified, Electron Microscopy Sciences, Washington, USA) in s-collidin buffer. After dehydration, embryos were embedded in epoxy resin and the sagittal sections were cut to a thickness of 1 μm. For analysis of mitotic nuclei, sections were stained with 0.5% toluidine blue.

Analyses of cellular proliferation

BrdU incorporation

BrdU incorporation assay was carried out using a "Cell Proliferation Kit" (Amersham-Pharmacia, RPN20) according to manufacturer's instructions. Pregnant females received intraperitoneal injection of BrdU at gestational days E9.5 and E10.5 and were sacrificed after 1 h. Sagittal and coronal paraffin sections were prepared as described above. We used 2N HCL, 0.01% trypsin treatment to denature paraffin sections, and reveal epitope prior to incubation with mouse monoclonal BrdU antibody.

Percentage of the proliferative cells in neuroectoderm was counted as (number of BrdU-positive cells/total number of cells) × 100. BrdU-positive cells were counted from four regions: rhombomere 1 region located at the distance from the boundary (R1), rhombomere 1 region adjacent to the midbrain–hindbrain boundary (R1B), midbrain region adjacent to the midbrain–hindbrain boundary (MBB), and midbrain region located at the distance from the boundary (MB) (see Figs. 6A and B). *Otx2* was used as a marker of the midbrain cells in the parallel sections. Forty-micrometer-wide regions, adjacent to the boundary (R1B and MBB) and at the distance of 90 μm (R1 and MB) of E9.5 embryos, and 50-μm-wide regions (R1B, R1, MBB, and MB) of E10.5 embryos were analyzed.

Mitotic nuclei

Metaphase nuclei were counted from sagittal semithin sections stained with toluidine blue. Cells, which showed condensed chromatin and lack of nucleolus, were counted as mitotic cells. Percentage of the mitotic cells was counted as (number of mitotic cells/total number of cells) × 100. The cells were counted from four 100-μm-wide and 10-μm-high adjacent regions next to the ventricle (R1, R1B, MBB, and MB; see Fig. 6D).

Immunohistochemistry for anti-phosphohistone-H3

For vibratome sections, embryos were fixed overnight in 4% PFA at +4°C and equilibrated in gelatin–albumin

mixture [100 ml 1× PBS, 0.49 g gelatine (Sigma), 30 g BSA (Sigma), 20 g sucrose (ICN-Biochemicals)]. For embedding, 880 μl of glutaraldehyde (25% solution, EM grade, Distillation Purified, Electron Microscopy Sciences) was added to 8 ml gelatine–albumin mixture and shortly vortexed. Equilibrated embryos were embedded onto the gelatine–albumin–glutaraldehyde mixture and sectioned sagittally at 70 μm. In all cases, sagittal sections around the midline were collected.

Sections were blocked for 3 h RT in TBSTD [1× TBS, 0.1% Triton-X, 5% DMSO (J.T.Baker) + 5% BSA (Sigma) + 0.4% sheep serum (Sigma)] and incubated in phosphohistone-H3 (Upstate) 1:800 in blocking solution for 48 h in +4°C. Sections were rinsed several times with TBSTD before incubated overnight at +4°C in secondary antibody 1:300 in blocking solution (anti-rabbit IgG, Alexa-488, Molecular Probes). Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain (Oriola cat H-1200). Sections were washed three times with TBSTD and mounted in Mowiol (Calbiochem). Immunostaining was visualized with a Biorad MRC 1024 Confocal microscope. Optical sections were combined using the Confocal Assistant 4.02 program.

Analyses of cell death

TUNEL analyses

TUNEL assays were performed on paraffin sections of E8.5 and E9.5 embryos with Fluorescein In Situ Detection Kit (Roche cat 1684 795). Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain (Oriola cat H-1200). *Otx2* was used as a marker of the midbrain cells in the parallel sections. At E8.5, apoptotic cells were counted in the region, marked with white lines, on both sides of the midbrain–hindbrain boundary (mb and r1). At E9.5 cells were counted from the 50-μm-wide region on both sides of the *Otx2* boundary.

In situ hybridization analyses

Whole-mount mRNA in situ hybridization analyses of E7.5–E10.5 day embryos with *Otx2* (Acampora et al., 1997), *Fgf8* (Crossley and Martin, 1995), *Fgf15* (McWhirter et al., 1997), *Wnt1* (a gift from Klaus Schughart), *Fgfr1* (Trokovic et al., 2003), *Fgfr2* (a gift from Alka Mansukhani), *Fgfr3* (Peters et al., 1993), *Spry1* (a gift from Seppo Vainio), *Erm* (IMAGE 3674281), *Pea3* (Lin et al., 1998), *CyclinD1* (IMAGE 3155470), *CyclinD2* (IMAGE 367058), *Jumonji* (IMAGE 6406875), and *p21* (a gift from Bert Vogelstein) were performed as described (Henrique et al., 1995). In situ hybridization on paraffin sections with *Fgfr1*, *Fgfr2*, *Fgfr3*, *Spry1*, *Fgf15*, *Fgf8*, *Wnt1*, *Otx2*, *p21*, *CyclinD2*, and *HoxA2* (a gift from Mario Capecchi) were carried out as described (Wilkinson and Green, 1990) using [^{35}S] labeled RNA probes.

Results

Initial changes in gene expression occur close to the midbrain–hindbrain boundary in Fgfr1 mutants

To study FGF signaling in midbrain–hindbrain development, we have generated mice in which one of the FGF receptors, *Fgfr1*, is inactivated in the midbrain and rhombomere 1 by tissue-specific mutagenesis. Mice homozygous for a conditional *Fgfr1* allele (*Fgfr1*^{Flox}) and heterozygous for an *En1-Cre* allele (*En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$) have abnormalities in midbrain and cerebellar development (Trokovic et al., 2003). To determine where the initial gene expression changes occur in *En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$ embryos, we analyzed the expression of downstream targets of FGF signaling pathway by whole-mount in situ hybridization on E9.5 embryos. *Pea3* and *Erm* are members of ETS family transcription factors and thought to be proximal targets of FGF signaling. Expression of *Pea3*, *Erm*, and *Sprouty1*, an FGF-inducible tyrosine kinase inhibitor (Minowada et al., 1999), was initially down-regulated close to the midbrain–hindbrain boundary in *En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$ embryos compared to the wild type (Figs. 1A–C). However, some expression of these genes was still detected further away from the midbrain–hindbrain border both in dorsal and ventral regions in the mutants. This suggests that a receptor tyrosine kinase other than FGFR1 regulates Spry1, Pea3, and *Erm* in the cells, which are located some distance away from the boundary. We also analyzed the expression of *Fgf15* and *Fgf8*. In wild-type embryos, *Fgf15* was expressed in the midbrain and rhombomere 1 of the hindbrain but not near their boundary in a region that coincides with the most rostral *Gbx2* expression (Gimeno, 2003). Interestingly, expression of *Fgf15* was up-regulated at the midbrain–hindbrain boundary in the mutant embryos compared to the wild type (Fig. 1D). At E9.5, *Fgf8* expression was not affected in *En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$ embryos compared to the wild type (Fig. 1E). However, at E10.5 *Fgf8* expression was clearly down-regulated at the midbrain–hindbrain boundary (Fig. 1F). These analyses of gene expressions thus suggested that initial changes in gene expression in *En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$ embryos occur close to the midbrain–hindbrain boundary.

Other FGFRs may mediate FGF signaling at a distance from the midbrain–hindbrain boundary

To understand why the downstream targets of FGF signaling are initially down-regulated at the boundary, but are still expressed away from it, we compared the expressions of *Fgfr1*, *Fgfr2*, and *Fgfr3* in E7.5–E9.5 wild-type embryos. Consistent with earlier studies (Ishibashi and McMahon, 2002; Liu et al., 2003; Trokovic et al., 2003) *Fgfr1* was found to be expressed in the head fold region of E7.5 embryos and throughout the neural tube of E8.5 and

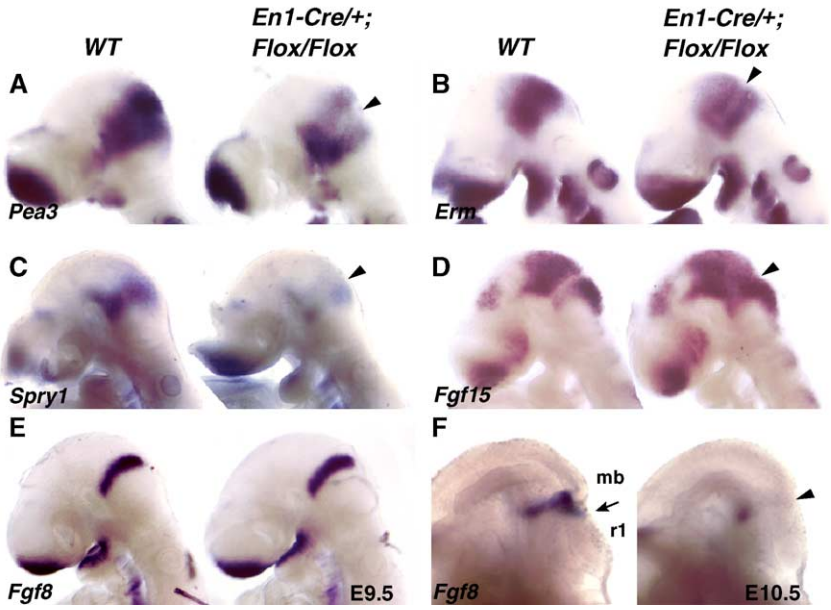

Fig. 1. Initial changes in gene expression occur close to the midbrain–hindbrain boundary in the *Fgfr1* mutants. Whole-mount in situ hybridization analysis of E9.5 wild-type and *En1-Cre/+* ;*Fgfr1*$^{Flox/Flox}$ embryos with *Pea3* (A), *Erm* (B), *Spry1* (C), and *Fgf15* (D). *Fgf8* expression was analyzed both at E9.5 (E) and E10.5 (F). Arrowheads indicate altered gene expression in mutant embryos. An arrow marks the midbrain–hindbrain boundary (F). See text for detailed description. mb, midbrain; r1, rhombomere 1.

E9.5 embryos (Fig. 2A; Supplementary Fig. S1A and D). *Fgfr2* was also expressed in the head fold region of E7.5 embryos but its expression was not detected at the midbrain–hindbrain boundary region of E8.5 and E9.5 embryos (Fig. 2B; Supplementary Fig. S1B and E). *Fgfr3* expression was not detected in the head fold region at E7.5 and was also absent from the midbrain–hindbrain boundary region of E8.5 and E9.5 embryos similar to the *Fgfr2* expression (Fig. 2C; Supplementary Fig. S1C and F). Thus, *Fgfr1* seems to be the only FGF receptor expressed at the

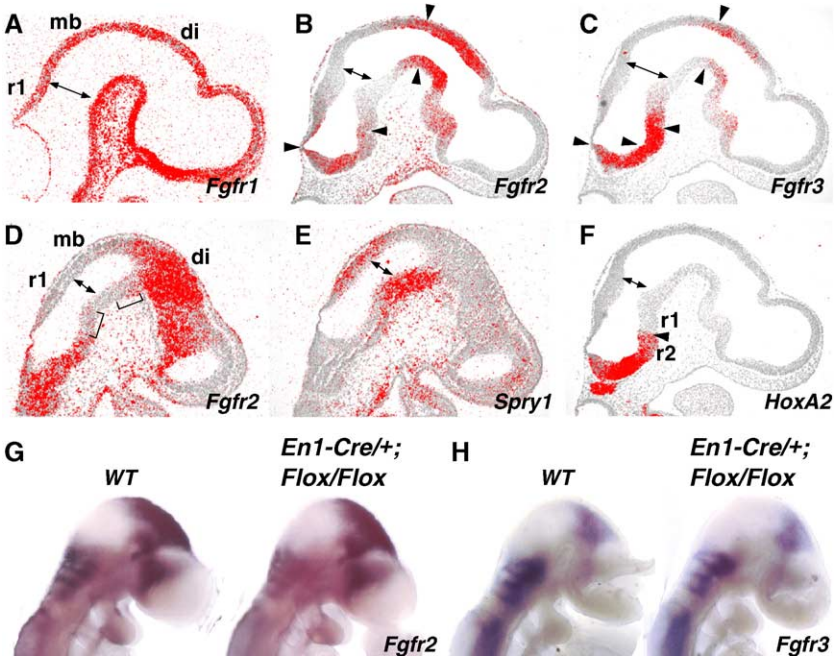

Fig. 2. Other FGFRs may receive IsO signals further away from the midbrain–hindbrain boundary. Section in situ hybridization analysis of *Fgfr1* (A), *Fgfr2* (B), and *Fgfr3* (C) expression at E9.5 (21–23 somite stage). A section adjacent to the ones shown in B and C hybridized with *HoxA2* probe (F). Arrowheads mark the midbrain–diencephalon and rhombomere 1/rhombomere 2 boundaries in B and C and rhombomere 1/rhombomere 2 boundary (F). Adjacent sagittal sections of E9.5 embryos hybridized with *Fgfr2* (D) and *Spry1* (E) probes show that *Fgfr2* and *Spry1* expressions overlap partly in the midbrain and rhombomere 1 (brackets in D). Whole-mount in situ hybridization analysis of *Fgfr2* (G) and *Fgfr3* (H) expression in E9.5 wild-type and *En1-Cre/+* ;*Fgfr1*$^{Flox/Flox}$ embryos. Arrows (A–F) mark the midbrain–hindbrain border. r1, rhombomere 1; r2, rhombomere 2; mb, midbrain; di, diencephalon.

boundary region whereas the expression of *Fgfr2* and *Fgfr3* in the midbrain–hindbrain region was graded gradually diminishing towards the boundary and could not be detected in the cells near the midbrain–hindbrain border by our in situ hybridization analyses. To study whether *Fgfr2* and *Fgfr3* are expressed in the rhombomere 1 of wild-type embryos at E9.5, we did in situ hybridization on serial adjacent sections with *Fgfr2*, *Fgfr3*, as well as *HoxA2*, a marker of rhombomere 2 (Figs. 2B, C, and F). Both *Fgfr2* and *Fgfr3* expressions were found to continue into the rhombomere 1. We further compared the expression patterns of *Spry1* and *Fgfr2* on adjacent sections. Interestingly, the expression of *Fgfr2* overlaps with the *Sprouty1* expression both in the rhombomere 1 and midbrain (Figs. 2D and E, brackets). Thus, FGFR2 and FGFR3 may also mediate FGF signaling in the midbrain and hindbrain, except for the boundary region.

We also examined the possibility that *Fgfr2* and *Fgfr3* expression could be up-regulated at the boundary region of *En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$ embryos. Whole-mount in situ expression studies with these genes in *En1-Cre/+*; *Fgfr1*$^{Flox/Flox}$ and wild-type embryos showed no difference in their expression patterns in the mid- and hindbrain region (Figs. 2G and H).

Isthmic constriction fails to develop in the Fgfr1 mutant embryos

One of the morphological landmarks of the boundary between midbrain and hindbrain is a constriction in the neural tube, which in the mouse embryos starts to form around E9.25. We analyzed the morphological development of the isthmic constriction in E10.5 and E11.5 *En1-Cre/+*; *Fgfr1*$^{Flox/Flox}$ embryos. In contrast to the wild-type embryos, in which the constriction is obvious at E10.5 and E11.5 (Figs. 3A, C, E, and G), the isthmic constriction was altered in *En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$ embryos at E10.5 and was absent at E11.5 (Figs. 3B, D, F, and H). These results further suggested that the cells at the boundary failed to develop normally.

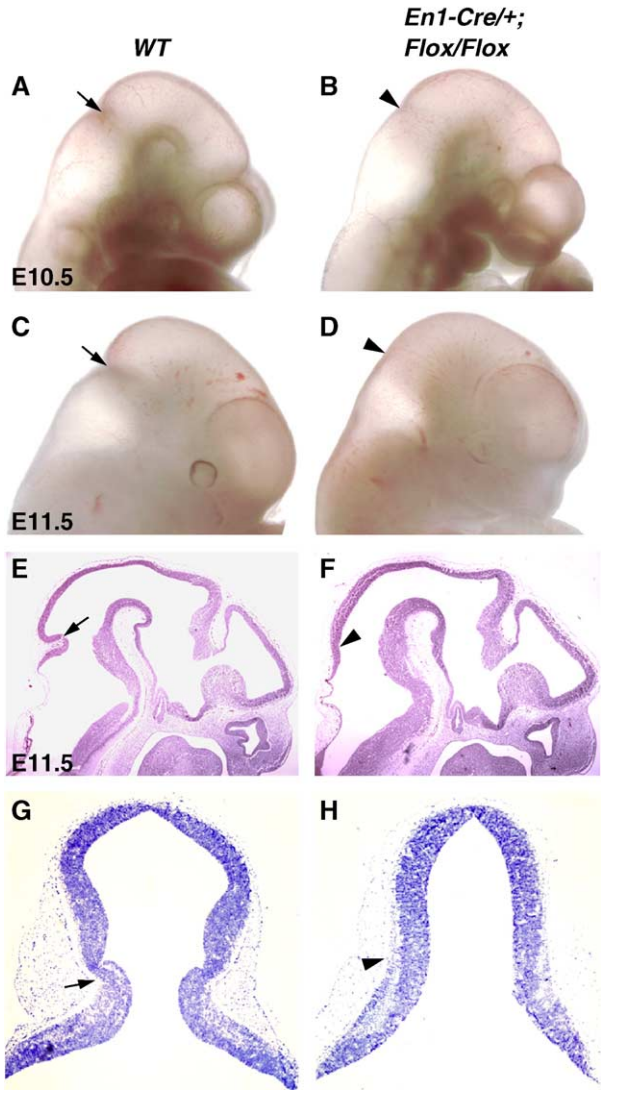

Fig. 3. *Fgfr1* mutants lack isthmic constriction. Lateral views of E10.5 (A and B) and E11.5 (C and D) embryos. Midsagittal (E and F) embryos and coronal (G and H) sections of E11.5 embryos. *En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$ mutants have defects in the isthmic constriction (arrowhead in B) at E10.5 compared to wild-type embryos (arrow in A). By 11.5 mutant embryos lack the isthmic constriction completely (arrowheads in D, F, and H) compared to wild-type embryos (arrows in C, E, and G).

Expression of cell cycle regulators at the midbrain–hindbrain boundary

The mechanisms behind the development of the isthmic constriction and properties of the cells at the boundary are poorly understood. To identify genes, which are expressed at the boundary, we have compared gene-expression profiles of wild-type and *En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$ embryos using microarrays (T.J., J.P., manuscript in preparation). Interestingly, several genes responsible for cell cycle regulation were differentially expressed in *En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$ embryos.

We found that both *CyclinD1* and *CyclinD2* were expressed in the midbrain–hindbrain region between E9.5 and E11.5. In the wild-type embryos, *CyclinD1* was expressed broadly in the midbrain and hindbrain at E10.0 (Fig. 4A; 30 somite stage). A negative *CyclinD1* domain was detected at the boundary. By contrast, in the *En1-Cre/+*; *Fgfr1*$^{Flox/Flox}$ embryos this domain was clearly absent (Fig. 4B). By E10.5 (36 somite stage), a negative domain in *CyclinD1* expression between midbrain and hindbrain had become more apparent in wild-type (Fig. 4C) but was completely missing in the *En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$ embryos (Fig. 4D). Similarly, *CyclinD2* was highly expressed in E10.5 wild-type embryos in the dorsal midbrain and hindbrain but there was a gap in expression at the midbrain–hindbrain boundary (Fig. 4E). In the *En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$ embryos, this domain of cells that did not express *CyclinD2* in the dorsal boundary region was no longer seen (Fig. 4F).

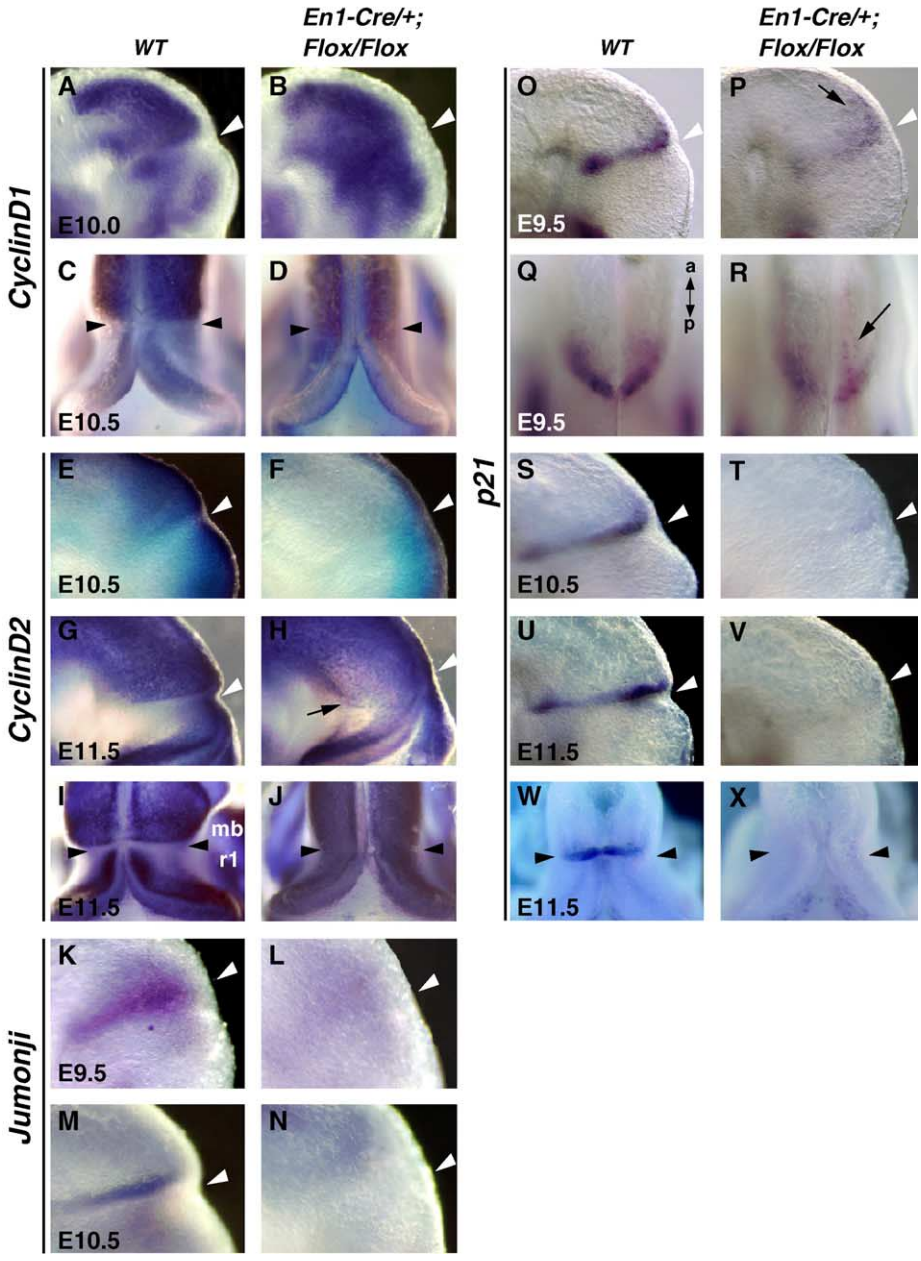

Fig. 4. Expression of cell cycle regulators at the midbrain–hindbrain boundary in wild-type and *Fgfr1* mutant embryos. Whole-mount in situ hybridization analysis of *CyclinD1* (A–D), *CyclinD2* (E–J), *Jumonji* (K–N), and *p21* (O–X) expression in wild-type and *En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$ embryos. Lateral views of the whole-mount-stained embryos at E9.5 (K, L, O, and P; 26–27 somite stage), E10.0 (A and B; 30 somite stage), E10.5 (E, F, M, N, S, and T; 36 somite stage), and E11.5 (G, H, U, and V), anterior leftwards. Dorsal views of the embryos at E9.5 (Q and R; 26–27 somite stage), E10.5 (C and D; 36 somite stage), and at E11.5 (I, J, W, and X), anterior towards the top. The midbrain–hindbrain boundary is marked by arrowheads. Arrow in H indicates the altered expression of *CyclinD2* at the ventral region. At E9.5, *p21*-positive cells are scattered in the mutant embryos (arrows in P and R) and do not form a narrow band at the boundary (arrowhead in O). Anterior–posterior axis is marked with a double-headed arrow (Q and R). mb, midbrain; r1, rhombomere 1.

On the other hand, the expression of *CyclinD2* in the midbrain was slightly down-regulated in the mutant embryos at E10.5. At E11.5, the negative gap in *CyclinD2* expression was more prominent in the wild-type embryos (Figs. 4G and I). The ventral border of *CyclinD2* expression was sharpened in the E11.5 wild-type embryos compared to the earlier stage. In the E11.5 *En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$ embryos, a diffuse *CyclinD2* expression was observed posterior to the boundary (Fig. 4H, arrow). The gap in *CyclinD2* expression was clearly absent in the mutant embryos (Figs. 4H and J). At an earlier stage (E9.5), *CyclinD1* and *CyclinD2* expressions were observed throughout the midbrain–hindbrain region (data not shown).

Jumonji (*Jmj*) is a transcription factor, which has recently been reported to directly negatively regulate *CyclinD1* expression and proliferation of developing cardiomyocytes (Toyoda et al., 2003). Consistent with earlier results (Takeuchi et al., 1995), we observed expression of *Jmj* as

a stripe at the midbrain–hindbrain boundary in wild-type embryos at E9.5 and E10.5 (Figs. 4K and M). In the *En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$ embryos, *Jmj* was clearly down-regulated at both stages (Figs. 4L and N). Thus, it is possible that *Jmj* negatively regulates *CyclinD1* expression also at the midbrain–hindbrain boundary.

In addition to the *Cyclins*, cyclin-dependent kinase (CDK) activity and cell cycle are also regulated by CDK inhibitors such as *p21* (Parker et al., 1995). We analyzed *p21* expression at the midbrain–hindbrain region at E9.0–11.5. A weak signal was observed in the ventral midbrain–hindbrain boundary region already at E9.0 (22–24 somite stage; data not shown). Later, we observed *p21* expression throughout the midbrain–hindbrain boundary both in E9.5 (26–27 somite stage) wild-type and *En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$ embryos. In the wild-type embryos, *p21* was expressed as a narrow stripe at the midbrain–hindbrain boundary (Figs. 4O and Q). In contrast, *p21*-positive cells did not form a coherent band of cells but were dispersed both in midbrain and rhombomere 1 in the *En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$ embryos (Figs. 4P and R, arrows). At the E10.5, when the isthmic constriction is already seen in the wild-type embryos, *p21* was expressed more prominently at the midbrain–hindbrain boundary (Fig. 4S). Its expression domain appeared to correspond to the region where *CyclinD1/2*-negative cells were observed. In the *Fgfr1* mutant embryos, *p21* expression was almost abolished at the E10.5 (Fig. 4T). By E11.5, the *p21* expression in the wild-type embryos was observed in the narrow stripe at the boundary (Figs. 4U and W) corresponding partially to *CyclinD2* negative domain (Figs. 4G and I). The *p21*-postive cells were not detected at E11.5 in the mutant embryos (Figs. 4V and X).

To further define the limits of expression of *CyclinD2* and *p21* at the midbrain–hindbrain region, we conducted in situ hybridizations on sections of E9.5 and E10.5 wild-type embryos (Fig. 5). To reveal the midbrain–hindbrain boundary itself, parallel sections were hybridized with *Fgf8*, *Otx2*, or *Wnt1* probes. Interestingly, we found that *p21* expression domain was observed both in the most anterior hindbrain (*Fgf8*-positive cells) and posterior midbrain (*Otx2*/*Wnt1*-positive cells) both at E9.5 and E10.5. The *p21* expression was detected only very close to the midbrain–hindbrain boundary and only partially overlapped with *Fgf8* expression in the rhombomere 1 (Figs. 5A and B) and *Otx2/Wnt1* expression in the posterior midbrain (Figs. 5C, D, G, and H). Similarly, a domain negative for *CyclinD2* expression was detected on both sides of the midbrain–hindbrain boundary at E10.5 (Fig. 5I). However, in the *En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$ embryos, *CyclinD2* expression was continuous across the midbrain–hindbrain boundary (Fig. 5J). We measured the width of the *p21* expression domain to be approximately 30 μm both at E9.5 and E10.5 (Figs. 5A, C, and 6A). By contrast, the *Fgf8* expression domains were found to be approximately 100 μm in width at same stages (Figs. 5B and 6A).

Cellular proliferation close to the midbrain–hindbrain border

Given the differential expression of cell cycle regulators close to the midbrain–hindbrain boundary, we wanted to compare the proliferative kinetics of these cells with the adjacent neural ectoderm in midbrain and rhombomere 1. To label the S-phase cells, E9.5 and E10.5 embryos were given a pulse of BrdU. Adjacent sagittal and coronal (E10.5) sections were analyzed either for BrdU incorporation to identify cells in the process of DNA replication, or *Otx2* expression to reveal the midbrain–hindbrain border (Fig. 6B; Supplementary Fig. S2). Proportion of BrdU-positive cells was counted in four regions of the neural epithelium shown in Fig. 6B: rhombomere 1 region located at a distance from the boundary (R1), rhombomere 1 region adjacent to the midbrain–hindbrain boundary (R1B), midbrain region adjacent to the midbrain–hindbrain boundary (MBB), and midbrain region located at a distance from the boundary (MB). The width of these regions used for counting the BrdU-positive cells at E9.5 and E10.5 were 40 and 100 μm, respectively. At E9.5, we were not able to see significant difference between these regions (Supplementary Fig. S2A, $n = 7$). However, at E10.5 there was a small but statistically very significant reduction in the BrdU incorporation indexes of both R1B and MBB compared to the R1 and MB (Figs. 6B and C, $n = 8$; Supplementary Fig. S2B and C).

To further characterize the proliferative properties of the cells at the boundary, sagittal semithin sections of E10.5 embryos were analyzed for the presence of the mitotic cells (Fig. 6D, $n = 8$). During cell cycle, the nuclei of early neuroectodermal cells move so that the mitoses take place close to the ventricular surface. We counted proportion of metaphase nuclei in four juxtaventricular regions: R1, R1B, MBB, and MB (Fig. 6D). The regions counted were 100 μm in width as in the BrdU incorporation analysis (E10.5). We found that there were significantly less mitotic cells in the R1B and MBB regions compared to the R1 and MB regions located further away from the boundary (Fig. 6E). We also analyzed mitotic cells by labeling them with anti-phosphohistone-H3 antibody. In wild-type E10.5 embryos, only few phosphohistone-H3-positive cells were observed close to the midbrain–hindbrain border (Fig. 6F, $n = 6$). In contrast, no reduction in phosphohistone-H3-positive cells at the boundary was not seen at the midbrain–hindbrain boundary of E10.5 *En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$ mutant embryos (Fig. 6G, $n = 5$). We also detected less mitotic cells in the boundary region of E11.5 wild-type embryos (Fig. 6H, $n = 6$).

Apoptosis close to the midbrain–hindbrain boundary

To study whether increased cell death could be responsible for the loss of *p21*-positive cells by E10.5 in *Fgfr1* mutants, we compared apoptotic cells of wild-

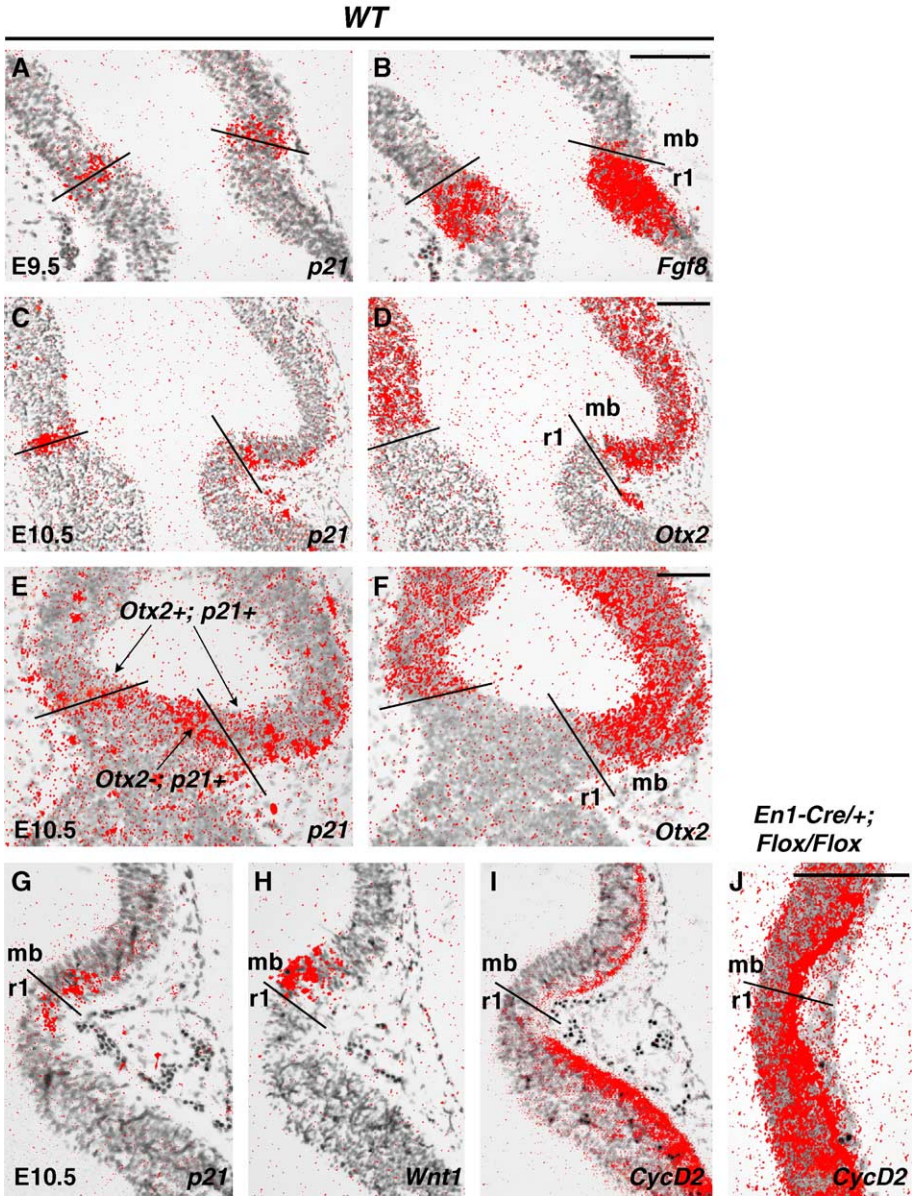

Fig. 5. Comparative expression analysis of *CyclinD2* and *p21* with hindbrain (*Fgf8*) and midbrain (*Otx2* and *Wnt1*) markers. Parallel sagittal sections of E9.5 wild-type embryos probed with *p21* (A) and *Fgf8* (B). Parallel sagittal (C and D) and para-sagittal (E and F) sections of E10.5 wild-type embryos hybridized with *p21* (C and E) and *Otx2* (D and F). Parallel sagittal sections of E10.5 wild-type embryos hybridized with the *p21* (G), *Wnt1* (H), and *CyclinD2* probes (I). A section from an E10.5 *En1-Cre/+* ; $Fgfr1^{Flox/FLox}$ embryo hybridized with a *CyclinD2* probe (J). The midbrain-hindbrain boundary is marked by a line. mb, midbrain; r1, rhombomere 1. Scale bars, 100 μm.

type and mutant embryos at E8.5 and E9.5. Cells were counted in two regions, R1B and MBB, both side of the midbrain–hindbrain boundary. The regions counted were 50 μm in width. Statistical analysis showed no difference between mutant and wild-type embryos (Supplementary Fig. S3).

Effect of Wnt signals on the p21-positive midbrain–hindbrain boundary cells

To study the possible collaboration between FGF and Wnt signaling in the induction of the boundary cell phenotype, we analyzed the effect of ectopic *Wnt1* expression on *p21*-positive boundary cells. For this, we used the $En1^{+/Wnt1}$ mice, in which *Wnt1* is expressed in a broad region of the midbrain as well as rhombomere 1 (Panhuysen et al., 2004). To determine whether excessive *Wnt1* signaling could expand the population of slowly proliferating cells at the boundary, we analyzed the expression of *p21* in $En1^{+/Wnt1}$ and wild-type embryos (Fig. 7). In the dorsal and lateral regions, the expression of p21 appeared to be similar in the $En1^{+/Wnt1}$ (Figs. 7B and D) and wild-type (Figs. 7A and C) embryos at E11.5. Interestingly, *p21* expression appeared to be up-regulated

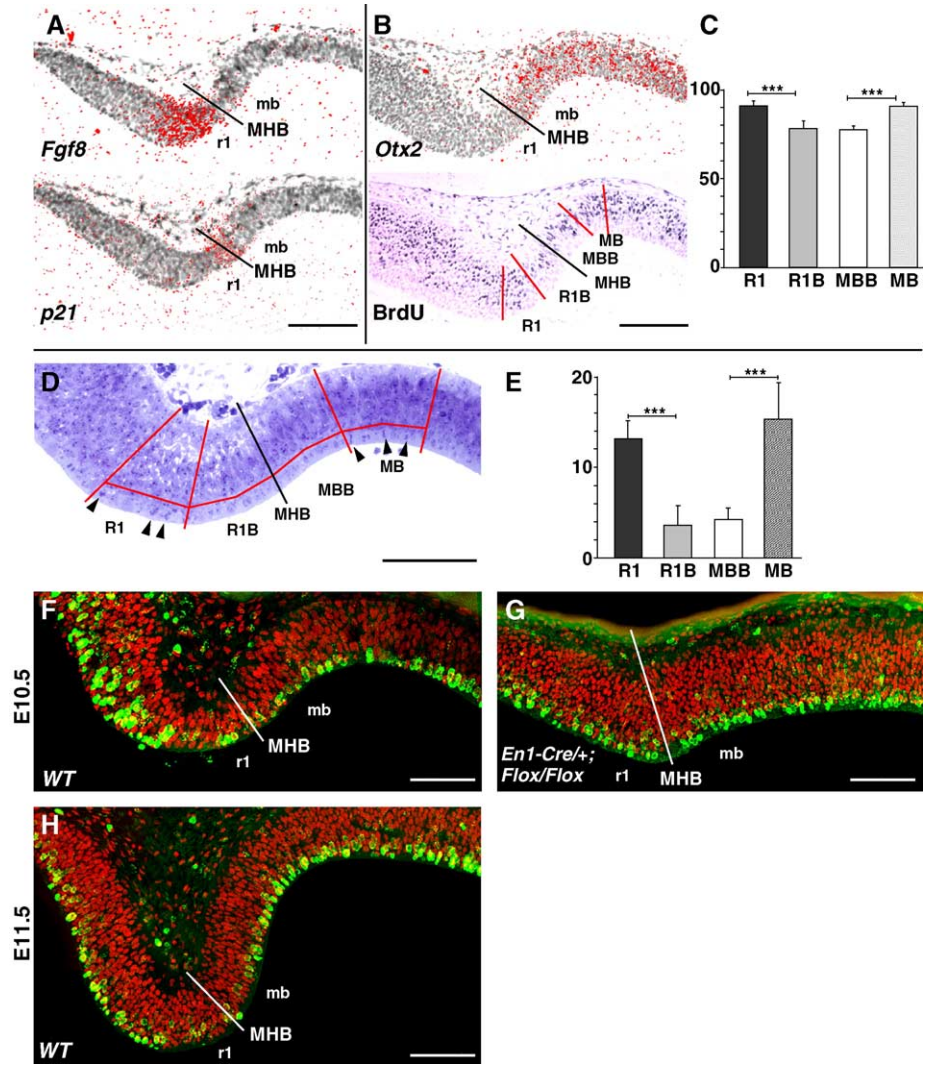

Fig. 6. Neuroepithelial proliferation in the midbrain–hindbrain boundary region. Adjacent sagittal sections of E10.5 embryos (A) were probed with *Fgf8*, revealing the midbrain–hindbrain boundary, and *p21*, revealing a specialized group of boundary cells. Adjacent sagittal sections of E10.5 (B) embryos were probed with *Otx2*, revealing the midbrain–hindbrain boundary, and anti-BrdU antibody, labeling the cells undergoing DNA replication, anterior rightwards. The areas of the neuroepithelium where BrdU incorporation was quantified (R1, R1B, MBB, and MB) are indicated, see text for details. Amount of mitotic cells was also measured from sagittal semithin sections of E10.5 mouse embryos (D), anterior rightwards. The quantified areas are similarly defined as above. The metaphase nuclei were counted in the juxtaventricular region where all the neuroepithelial cell mitoses take place at this stage. Anti-phosphohistone-H3 immunostaining of sagittal sections of E10.5 wild-type (F), E10.5 *En1-Cre/+*;*Fgfr1*$^{Flox/Flox}$ (G), and E11.5 wild-type (H) embryos. Each bar presents data from seven embryos (C and E). Error bar represents standard deviation. Statistical analysis was performed using Student's *t* test (***$P < 0.001$). mb, midbrain; r1, rhombomere 1. Scale bars = 100 μm.

in the most ventral region of the midbrain–hindbrain boundary of the *En1*$^{+/Wnt1}$ embryos (Fig. 7B, arrow).

Discussion

Maintenance of a coherent signaling source is essential for organizer activity. We show here that at the boundary between developing midbrain and rhombomere 1, there is a narrow population/populations of cells with distinct gene expression and cell cycle characteristics. These cells require signaling through *Fgfr1* to maintain their integrity. Defect in these cells appears to result in a failure to maintain a coherent border between midbrain and rhombomere 1.

FGF signaling from the IsO is generally thought to stimulate proliferation and suppress neuronal differentiation in adjacent neuroectoderm (Xu et al., 2000). Our results suggest that in addition of stimulating cellular proliferation, FGF signaling is required for establishment of a more slowly proliferating cell population in the middle of the proliferative region. We do not know if FGFR1 signaling is directly involved in negative regulation of cell cycle at the midbrain–hindbrain boundary. However, this would not be completely unprecedented as such growth inhibitory function has previously been associated with FGF signaling in regulation

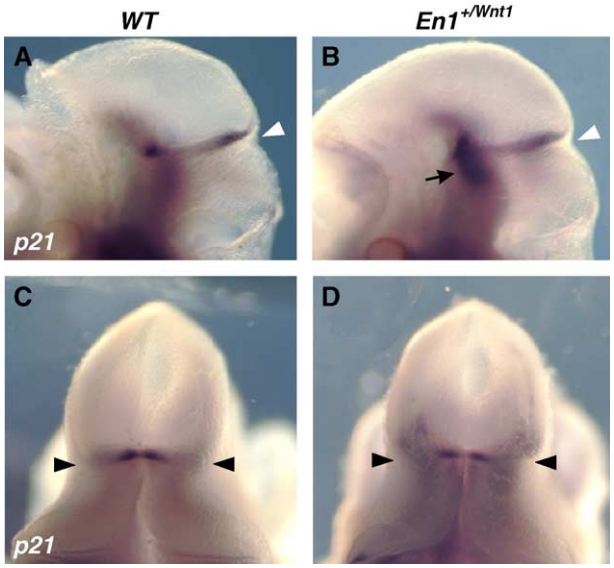

Fig. 7. Effect of Wnt signals on the *p21*-positive midbrain–hindbrain boundary cells. Whole-mount in situ hybridization analysis of the expression of *p21* in wild-type (A and C) and $En1^{+/Wnt1}$ (B and D) embryos at E11.5. Arrowheads mark the midbrain–hindbrain boundary. An arrow shows the altered expression in the most ventral region.

of chondrocyte proliferation (Dailey et al., 2003). Also in developing central nervous system, FGF signaling through FGFR1 negatively regulates cellular proliferation in the anterior forebrain resulting in the morphogenetic process of olfactory bulb evagination (Hebert et al., 2003).

Reduced cellular proliferation has been connected to the IsO also previously. Short survival thymidine autoradiography studies in E14 rat embryos have shown that the concentration of proliferative cells is higher in the cerebellar neuroepithelium than in the adjacent isthmus region (Altman and Bayer, 1997). These studies also showed that cerebellar neuroepithelium differs from the adjacent isthmal epithelium in the concentration of the metaphase cells. In addition, it has been shown that cells expressing *Fgf8* at the isthmus proliferate slower than cells in adjacent regions in E12.5 mouse embryos (Li et al., 2002). However, the isthmal cells appear to be different from the boundary cell populations described here. In the studies mentioned above, the area of slower cellular proliferation is observed to contain the entire *Fgf8*-positive isthmal region (100 μm in width at E10.5; Fig. 6A). In contrast, we detected reduced cell proliferation rate and *p21* expression only in the most anterior part of the *Fgf8* domain as well as in the posterior midbrain (Fig. 8). A likely explanation for these differences is the timing of analyses. In fact, Altman and Bayer (1997) observed abundant proliferation in the isthmus at earlier stages in the rat (E13, corresponding to E11.5 in mouse). Furthermore, in the thymidine autoradiograms of E13 rat, embryos presented by Altman and Bayer (1997) one can observe a small domain of reduced cellular proliferation, which appears to correspond well to the slowly proliferating midbrain–hindbrain boundary cells characterized here.

How does FGF signaling regulate the boundary cells? Our results suggest that *Fgfr1* is not absolutely required for the development of these cells, as *p21*-positive cells were still observed in E9.5 *Fgfr1* mutant embryos. However, the cells were spread out and failed to form a tight band at the boundary. It is possible that signals through FGFR1 affect the adhesive characteristics of these cells, perhaps by regulating expression of specific cell adhesion molecules, such as *PB-cadherin*. At E10.5, *p21*-positive cells could not been detected anymore in the *Fgfr1* mutant embryos. As we have not been able to detect increased cell death close to the boundary in the mutants (see Supplementary Fig. S3), it is

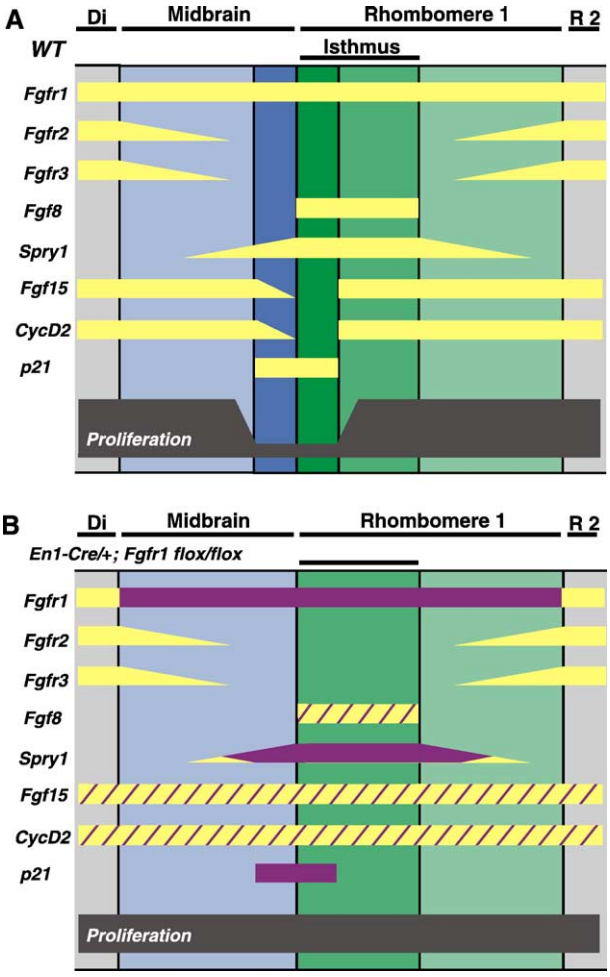

Fig. 8. Model of *Fgfr1*-dependent boundary cells. Gene expression and proliferative kinetics at the midbrain–hindbrain boundary in wild-type (A) and $En1\text{-}Cre/+;Fgfr1^{Flox/Flox}$ (B) embryos. Unlike the cells at a distance from the boundary, the cells close to the midbrain–hindbrain boundary (as determined by *Otx2* expression border) are dependent on FGFR1 for their response to the FGF signals. These boundary cell populations are negative for *CyclinD2* expression, express cell cycle inhibitors such as *p21*, and proliferate less rapidly than the surrounding cells. In the *Fgfr1* mutants the slowly proliferating boundary cell population is lost. This correlates with early down-regulation of *p21* expression and absence of *CyclinD2* and *Fgf15*-negative region at the midbrain–hindbrain boundary of the $En1\text{-}Cre/+;Fgfr1^{Flox/Flox}$ embryos. The purple color represents loss of gene expression. Hatching represents gradual down-regulation of *Fgf8*, *CyclinD2*, and *Fgf15* after E9.5 in the mutants.

possible that the dispersed boundary cells adopt fates characteristic to the more distal cells in the midbrain and rhombomere 1.

As the boundary cells are restricted to a narrow region in the most posterior midbrain and most anterior hindbrain in wild-type embryos, FGF signaling likely interacts with other regulators to promote the boundary cell phenotype. We have here analyzed possible interaction between FGF and Wnt signaling. Although *p21* expression was up-regulated in the most ventral part of the neural tube, the expression in dorsal and lateral regions appeared unaltered in transgenic embryos expressing ectopic *Wnt1*. This suggests that additional factors also contribute to the development of the boundary cell population.

What could be the significance of the boundary cells for the developing brain? They appear to be important for formation of the morphological constriction near the boundary between midbrain and hindbrain. Cellular mechanisms responsible for development of the isthmic constriction are poorly understood. It is possible that reduced cellular proliferation at the boundary contributes to this process. Although the function of the constriction is unclear, its development may correlate with compartmentalization of the midbrain and hindbrain (Trokovic et al., 2003; Zervas et al., 2004). Specialized cells have been associated with developmental compartment boundaries for example in the Drosophila wing imaginal discs as well as in more caudal regions of the hindbrain (Dahmann and Basler, 1999; Guthrie and Lumsden, 1991; Heyman et al., 1995). Furthermore, the rhombomere boundaries within the hindbrain have also been shown to be sites of reduced cellular proliferation (Guthrie et al., 1991), although the mechanisms of cell cycle regulation may be different. Our results suggest that the boundary region between mid- and hindbrain is divided into smaller cellular populations. Information on the properties and lineage contributions of these cells can be expected to be highly important for understanding isthmic organizer function and development of these brain regions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.11.024.

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