Gbx2 and Fgf8 are sequentially required for formation of the midbrain-hindbrain compartment boundary

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SUMMARY
In vertebrates, the common expression border of two homeobox genes, Otx2 and Gbx2, demarcates the prospective midbrain-hindbrain border (MHB) in the neural plate at the end of gastrulation. The presence of a compartment boundary at the MHB has been demonstrated, but the mechanism and timing of its formation remain unclear. We show by genetic inducible fate mapping using a Gbx2CreER knock-in mouse line that descendants of Gbx2+ cells as early as embryonic day (E) 7.5 do not cross the MHB. Without Gbx2, hindbrain-born cells abnormally populate the entire midbrain, demonstrating that Gbx2 is essential for specifying hindbrain fate. Gbx2+ and Otx2+ cells segregate from each other, suggesting that mutually exclusive expression of Otx2 and Gbx2 in midbrain and hindbrain progenitors is responsible for cell sorting in establishing the MHB. The MHB organizer gene Fgf8, which is expressed as a sharp transverse band immediately posterior to the lineage boundary at the MHB, is crucial in maintaining the lineage-restricted boundary after E7.5. Partial deletion of Fgf8 disrupts MHB lineage separation. Activation of FGF pathways has a cell-autonomous effect on cell sorting in midbrain progenitors. Therefore, Fgf8 from the MHB may signal the nearby mesencephalic cells to impart distinct cell surface characteristics or induce local cell-cell signaling, which consequently prevents cell movements across the MHB. Our findings reveal the distinct function of Gbx2 and Fgf8 in a stepwise process in the development of the compartment boundary at the MHB and that Fgf8, in addition to its organizer function, plays a crucial role in maintaining the lineage boundary at the MHB by restricting cell movement.

KEY WORDS: Gbx2, Otx2, Fgf8, Transcription factor, Compartment, Lineage boundaries, Mouse, Chick

INTRODUCTION
A fundamental mechanism for coordinating the growth and patterning of cellular fields in both invertebrate and vertebrate embryos is the process of segmentation, whereby cell mixing is inhibited at a compartment boundary (Kiecker and Lumsden, 2005). A key function of compartment boundaries is to prevent intermixing of cells that are destined for different developmental fates. Furthermore, cells near the lineage restriction boundary often serve as a signaling center by producing secreted factor(s) that control the development of cells flanking the compartment boundary (Irvine and Rauskolb, 2001). How compartment boundaries are established and maintained in vertebrate embryos remains to be elucidated.

The midbrain and the cerebellum are derived from two developmental compartments in the neural tube called the mesencephalon (mes) and rhombomere 1 (r1), respectively. After the neural tube closes, a constriction called the isthmus is formed near the junction between the mes and r1 in the mouse embryo at embryonic day 9.5 (E9.5). However, as early as E7.5, the prospective midbrain-hindbrain border (MHB) may already be defined by a common border of the expression domains of two homeobox genes, Otx2 and Gbx2, in the neural plate (Joyner et al., 2000). Reciprocal inhibition between Otx2 and Gbx2 results in mutually exclusive expression of both genes in the neural plate and defines the position of the prospective MHB (Broccoli et al., 1999; Katahira et al., 2000; Li and Joyner, 2001; Millet et al., 1999). Deletion of Otx2 or Gbx2 in mice results in loss of the midbrain or the cerebellum, respectively, demonstrating the essential role of these genes in the development of these brain structures (Acampora et al., 1998; Rhinn et al., 1998; Wassarman et al., 1997). Between E8.0 and E8.5 two secreted molecules, Wnt1 and Fgf8, are induced in mes (Otx2+) and r1 (Gbx2+) cells, respectively, and the expression domains of Wnt1 and Fgf8 become restricted to two narrow transverse bands immediately adjacent to the MHB by E9.5 (Liu and Joyner, 2001). A study using genetic inducible fate mapping (GIFM) with Wnt1-CreER transgenic mice demonstrated that the MHB is a lineage-restricted boundary (Zervas et al., 2004). This study, however, showed that the lineage boundary at the MHB is established between E8.5 and E9.5. Important questions to be resolved are whether the lineage boundary at the MHB is established earlier and if it is linked to heritable expression of Otx2 and Gbx2 in the mouse embryo.

Embryological and genetic experiments have demonstrated that Fgf8 is the key molecule of a signaling center, called the isthmic organizer, at the MHB (Chi et al., 2003; Crossley et al., 1996; Martinez et al., 1999; Shamim et al., 1999). In the absence of Fgfr1, which encodes a receptor for Fgf8, clusters of cells of r1 or mes characteristics are present in the mes and r1 regions, respectively, indicating that fibroblast growth factor (FGF) signaling is essential for the lineage boundary at the MHB (Trokoivic et al., 2005; Trokoivic et al., 2003). However, it remains unknown whether the MHB phenotype of Fgfr1 mutants results from abnormal gene regulation or actual cell mixing at the MHB. If the latter is the case, how FGF signaling regulates boundary formation is unknown.

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In this study, we investigated the molecular and cellular mechanisms that regulate MHB formation. We examined the developmental fate of Gbx2-expressing cells at E7.5 using GIFM with a Gbx2 CreER knock-in mouse line. We extended our fate-mapping studies to embryos that lose Gbx2 or Fgfl between E7.5 and E9.5. By combining GIFM, chimeric and genetic mosaic studies, we have uncovered sequential roles of Gbx2 and Fgfl in the development of the MHB. Finally, we show that FGF signaling is not only required but also sufficient for regulating cell sorting in the mes region.

MATERIALS AND METHODS

Mouse breeding and genotyping

All mouse strains were maintained on a CD1 mixed genetic background. Noon of the day on which a copulatory plug was detected was designated as E0.5. For CreER-mediated genetic fate mapping and deletion, 4 to 6 mg tamoxifen (Sigma, St Louis, MO) in corn oil was administered to pregnant females by oral gavage using feeding needles. To delete Gbx2 or Fgfl between E7.0 and E8.0, tamoxifen was administered around 6:00 pm of E6.5 (designated as E6.75).

Mice carrying the Gbx2 CreER allele were identified by PCR or enhanced green fluorescent protein (EGFP) fluorescence (Chen, L. et al., 2009). Other alleles were genotyped as previously described: Gbx2+/− (Li et al., 2002); Fgfl−/− and Fgfl+/− (Sun et al., 2002); R26RlacZ and R26R+/− (Soriano, 1999). CreER-mediated recombination in Gbx2 CreER+/−; R26R lacZ−/− or Fgfl−/−; Gbx2 CreER+/−; R26R lacZ−/− embryos that received tamoxifen between E6.5 and E8.5 was determined by 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) histochemistry of the tail. Embryos with strong X-gal staining (high percentage of recombination) were collected for in situ hybridization. Generation of Gbx2-deficient embryonic stem (ES) cells and chimera analysis were performed as described previously (Chen, L. et al., 2009).

Histological and immunohistochemical analyses

RNA in situ hybridization on sections or in whole-mount immunohistology, and β-galactosidase (β-gal) histochemistry were performed as described previously (Chen, L. et al., 2009), and detailed protocols for these assays are described on the Li lab website (http://llab.uchc.edu/Pages/Protocols.html). The following antibodies were used: rabbit anti-GFP IgG and mouse anti-TuJ1 (Covance). The following antibodies were used: rabbit anti-GFP IgG (Sigma, St Louis, MO), and mouse anti-Otx2 (1:150, Covance). The following antibodies were used: rabbit anti-GFP IgG (Sigma, St Louis, MO), and mouse anti-Otx2 (1:150, Covance). The following antibodies were used: rabbit anti-GFP IgG (Sigma, St Louis, MO), and mouse anti-Otx2 (1:150, Covance).

RESULTS

Analysis of the expression of Otx2 and Gbx2 at the MHB

To examine the cell fate of Gbx2-expressing cells, we recently generated a Gbx2 knock-in allele, Gbx2 CreER, in which a CreER-ires-Egfp cassette was inserted into the 5’ UTR of Gbx2 so that both CreER and EGFP were simultaneously expressed from the Gbx2 locus (Chen, L. et al., 2009). In Gbx2 CreER/+ embryos, EGFP fluorescence and Gbx2 transcripts were detected throughout the posterior part of the embryo and the prospective r1 at E7.5 and E8.5 (Fig. 1A,B and data not shown). To determine the anterior limit of EGFP expression in Gbx2 CreER/+ embryos, we performed double immunostaining using antibodies against EGFP and Otx2. Otx2+ and EGFP+ domains were complementary in the neural plate at E8.5 (Fig. 1C) and were juxtaposed at the isthmus at E9.5 (Fig. 1D). Given the mutual inhibition between Otx2 and Gbx2, we were surprised to detect EGFP in Otx2− cells in the ventral midline of the mes of Gbx2 CreER/+ embryos at E9.5 and E10.5 (Fig. 1G and see Fig. S1A in the supplementary material). Careful examination revealed the presence of Gbx2 transcripts in the ventral mes at E10.5, suggesting that the EGFP expression represents a previously unknown domain of Gbx2 expression in the ventral midline of the mes instead of ectopic expression of EGFP from the Gbx2 CreER allele (see Fig. S1A-C in the supplementary material). Therefore, the expression of EGFP recapitulates the endogenous Gbx2 expression in Gbx2 CreER/+ embryos.

We next used EGFP as a short-term lineage tracer to determine if Gbx2-expressing cells are restricted from crossing the MHB. We performed confocal imaging on flat-mount preparations of the mes-r1 region following immunostaining of Otx2 and EGFP in whole-mount Gbx2 CreER/+ embryos at E9.5 (Fig. 1D). Otx2+ cells and EGFP+ cells were mostly separated along the MHB with few EGFP+ cells in the mes, suggesting that Gbx2-expressing cells may be restricted from crossing the MHB between E7.5 and E9.5 (Fig. 1E,F). By contrast, many Otx2+/EGFP+ cells were found in r1 (Fig. 1E,F,F′). Significantly, these Otx2+/EGFP+ cells in r1 were positive for β-III tubulin (TuJ1), a postmitotic neuronal marker (Fig. 1H-J). Our data suggest that, although the mes and r1 progenitor cells are mainly segregated from each other at the MHB, some mes cells contribute to the hindbrain after they exit the cell cycle.

Gbx2-expressing cells and their descendants are restricted from crossing the MHB from E7.5 onward

To determine the fate of Gbx2-expressing cells, we combined the Gbx2 CreER allele and a Cre reporter allele, R26RlacZ (Soriano, 1999), and performed GIFM by administering tamoxifen to pregnant females carrying Gbx2 CreER+; R26R lacZ/− embryos at E6.75. Because tamoxifen induces CreER-mediated recombination in a 6 to 36 hour window (Joyner and Zervas, 2006), we deduced that giving tamoxifen at E6.75 would label Gbx2-expressing cells between E7.0 and E8.25. Analysis of β-gal activity by whole-mount histochemistry showed that marked descendants of the initial Gbx2-expressing cells were scattered throughout the posterior two-thirds of the embryo at E8.5, but absent from the prospective prosencephalon and mes (n=5) (Fig. 2A). Between E9.5 and E14.5, the vast majority of marked descendants of Gbx2-expressing cells were found posterior to the isthmus in Gbx2 CreER+; R26R lacZ/− embryos that were given tamoxifen at E6.75 (Fig. 2B,C, Fig. 3A,C). Therefore, the lineage boundary at the MHB is established around E7.5, and r1 cells are restricted from crossing this border until at least E14.5.

To determine if Gbx2-expressing cells at later stages are also restricted from crossing the MHB, we gave tamoxifen to females carrying Gbx2 CreER+; R26R lacZ/− embryos at E8.5 or E10.5. The anterior limit of marked descendants of the Gbx2 lineage was juxtaposed with the posterior limit of Otx2− domain at E10.5 and E18.5, except for a streak of fate-mapped cells in the ventral midline of the midbrain (Fig. 2D-G). The fate-mapped cells in the ventral midline of the midbrain are probably derived from the newly discovered region of Gbx2 expression in the ventral mes (Fig. 1J). Therefore, the Gbx2-lineage is mostly prevented from crossing the MHB between E7.5 and E14.5.
Gbx2 is required to specify r1 compartment identity

Gene expression studies have suggested that r1 cells may be transformed into a midbrain fate in Gbx2-deficient mice, yet definitive proof is still lacking (Li and Joyner, 2001; Millet et al., 1999; Wassarman et al., 1997). Taking advantage of the normal Gbx2 transcription in Gbx2-deficient embryos before E7.75 (Li and Joyner, 2001), we labeled the initial Gbx2-transcribing cells by administering tamoxifen at E6.75 to females carrying Gbx2CreER/−; R26RlacZ/+ embryos. The cerebellar primordium was absent in Gbx2CreER/−; R26RlacZ/+ embryos as found in Gbx2−/− embryos, demonstrating that Gbx2CreER is a null allele (Fig. 3B,D). Significantly, descendants of Gbx2-transcribing cells were found throughout the midbrain in Gbx2CreER/−; R26RlacZ/+ embryos (Fig. 3B,D). Moreover, the marked cells expressed Otx2 and intermixed with midbrain cells in Gbx2CreER/−; R26RlacZ/+ embryos at E12.5 following tamoxifen gavage at E6.75, demonstrating that r1 cells are transformed into a midbrain fate, which accounts for the loss of the cerebellum in Gbx2-deficient mice (Fig. 3D). Therefore, Gbx2 is essential for specifying the fate of r1 cells and for establishing a lineage-restriction boundary at the MHB.

Fig. 2. Descendants of Gbx2-expressing cells are restricted posterior to the MHB.

(A-C) X-gal histochemistry of Gbx2CreER/−; R26RlacZ/+ embryos at 8.5 (A) and 9.5 (B,C) following tamoxifen administration at E8.75. The arrowhead indicates the MHB. (D-F) X-gal histochemistry of whole-mount (D) and sagittal section of E10.5 Gbx2CreER/−; R26RlacZ/+ embryos (E), and horizontal section of E12.5 embryos (F) that received tamoxifen at E8.5. The inset in E shows in situ hybridization of Otx2 transcripts on sagittal section of E10.5 embryo. Note that X-gal+ cells are largely restricted to areas posterior to the caudal limit (dashed line) of the Otx2 expression domain, except for the ventral midline of the midbrain (arrow). The inset in F illustrates the plane of section (red line). (G,H) X-gal histochemistry (G) and in situ hybridization of Otx2 transcripts (H) on sagittal sections of Gbx2CreER/−; R26RlacZ/+ embryos at E18.5 after tamoxifen gavage at E10.5. cb, cerebellum; ic, inferior colliculus; ov, otic vesicle; sc, superior colliculus; tg, trigeminal ganglion.
Gbx2* and Otx2* cells mutually segregate from each other

Next, we performed chimera analysis to investigate the cell-autonomous requirement for Gbx2 in the establishment of the MHB. Chimeric embryos that were composed of mutant and wild-type cells were generated by microinjection of Gbx2-deficient ES cells (Gbx2*+/wild-type chimeras), or Gbx2*–/– ES cells (Gbx2*–/– wild-type chimeras) into blastocysts carrying the ROSA26 transgene, which expresses β-gal ubiquitously (Friedrich and Soriano, 1991). In chimeras, the host cells, which are phenotypically normal, are stained blue on X-gal histochemistry and are thus readily distinguished from ES-derived cells. Gbx2*–/– wild-type chimeras were morphologically normal, and Gbx2*–/– cells were distributed evenly throughout the embryos (Fig. 4A,C). By contrast, in Gbx2*–/– wild-type chimeras, the neuroepithelium of r1 displayed abnormal bulges and folds, and the neural tube often failed to close in the MHB region of chimeras with significant contribution of ES cells (>40%, data not shown). Although Gbx2*–/– cells normally mixed with wild-type cells in the forebrain, midbrain and posterior hindbrain of Gbx2*–/– wild-type chimeras, the mutant cells segregated from wild-type cells in the anterior hindbrain (Fig. 4B,D,E,G), demonstrating that Gbx2 is required cell autonomously for cell mixing in r1.

The mutual repression between Otx2 and Gbx2 prompted us to examine whether Otx2 was induced in Gbx2*–/– cells in the anterior hindbrain of Gbx2*–/– wild-type embryos. By comparing X-gal and Otx2 expression on adjacent horizontal sections of Gbx2*–/– wild-type embryos at the five-somite stage, E10.5, and E18.5, we found that Otx2 was expressed throughout the aggregates of Gbx2-deficient cells in the anterior hindbrain, demonstrating that Gbx2 is required cell-autonomously to inhibit Otx2 in r1 cells (Fig. 4D,F,H). The sorting of mutant (Otx2*) and wild-type host (Gbx2*) cells in chimeras suggests that the differential expression of Otx2 and Gbx2 may contribute to the segregation of mes and r1 cells in the neural plate in the establishment the lineage boundary at the MHB.

Mosaic deletion of Gbx2 results in abnormal cell sorting at the MHB

In Gbx2*–/– wild-type embryos, there appeared to be an accumulation of Gbx2-deficient cells in the posterior mes, consistent with a scenario in which Gbx2-deficient cells close to the MHB may sort into the mes (Fig. 4G). To test this model, we employed simultaneous ‘self-deletion’ and fate mapping by combining the Gbx2CreEr allele with a conditional mutant allele, Gbx2*locZ–/– (Gbx2*) and administering tamoxifen to Gbx2CreEr/F; R26RlacZ/+ embryos at E6.75 (Chen, L. et al., 2009; Li et al., 2002). In this experimental setup, the activation of CreER should convert the genotype of Gbx2-expressing cells from Gbx2CreEr/F (phenotypically normal) to Gbx2CreEr/F; Gbx2*locZ–/– (Gbx2 deficient). The recombined cells have a high probability of being marked by β-gal in Gbx2CreEr/F; R26RlacZ/+ mice.

In Gbx2CreEr/F; R26RlacZ/+ embryos that were given tamoxifen at E6.75, the alar plate of r1 was noticeably enlarged (Fig. 5H). Similar to that found in Gbx2*–/– wild-type chimeras, the neuroepithelium in posterior r1 was abnormally folded in Gbx2CreEr/F; R26RlacZ/+ embryos, suggesting mosaic deletion of Gbx2 in r1 cells (Fig. 5H inset and Fig. 5I). Although the labeled r1 cells formed a clear anterior border, this border had apparently shifted rostrally in Gbx2CreEr/F; R26RlacZ/+ embryos at E10.5 and E12.5 (Fig. 5C,D,H,J). To define the anterior limit of labeled cells with respect to the MHB, we performed X-gal histochemistry or double labeling of yellow fluorescent protein (YFP) and Otx2 on adjacent sections of E12.5 Gbx2CreEr/F; R26RlacZ/YFP embryos that were given tamoxifen at E6.75. Many labeled cells were found in the posterior part of the mes and expressed Otx2 (Fig. 5I). Therefore, r1 cells deficient for Gbx2 indeed sort into the mes compartment.

To verify mosaic deletion of Gbx2 and to examine altered gene expression due to the loss of Gbx2, we performed in situ hybridization on E9.5 Gbx2CreEr/F; R26RlacZ/+ embryos that received tamoxifen at E6.75. Using an RNA probe corresponding to Gbx2 exon II, which is deleted by Cre-mediated recombination (Li et al., 2002), we found that the level of Gbx2 expression was markedly reduced in all Gbx2-expressing tissues in Gbx2CreEr/F; R26RlacZ/+ embryos at E9.5, demonstrating mosaic deletion of Gbx2 in these embryos (Fig. 6B). Fgf8 is normally expressed in a tight stripe corresponding to the isthmus, whereas Wnt1 is expressed in a transverse ring immediately anterior to Fgf8 and in the dorsal midline of the neural tube, except for in the telencephalon and r1 (Fig. 6D,G). In Gbx2CreEr/F; R26RlacZ/+ embryos given tamoxifen at E6.75, the expression of Fgf8 was abnormally expanded throughout r1 (Fig. 6E); ectopic clusters of Wnt1-expressing cells were detected in the dorsal midline of r1 (Fig. 6H). Moreover, there were ectopic patches of Otx2* cells in caudal r1 and a slight expansion of Otx2 expression in the dorsal midline of r1 (Fig. 6K). Despite the anomalous expression within r1, the expression border of Fgf8, Wnt1 and Otx2 was mostly intact at the MHB. Therefore, in the mosaic loss of Gbx2, the lineage boundary of Gbx2 is shifted rostrally, and a new MHB is established behind the anterior limit of the Gbx2-lineage in Gbx2CreEr/F; R26RlacZ/+ embryos that received tamoxifen at E6.75.

To further investigate the requirement of Gbx2 in the maintenance of the lineage boundary at the MHB, we administrated tamoxifen to Gbx2CreEr/F; R26RlacZ/+ embryos at E8.5. No
significant defects were detected at the MHB \( (n=9, \text{see Fig. S2B in the supplementary material})\). Collectively, our results demonstrate that although Gbx2 is essential for the establishment of the MHB, the dependence on Gbx2 for continued stability of the boundary wanes at later stages. Therefore, additional mechanisms must be involved in maintaining the compartment boundary at the MHB.

**Fgf8 is essential for the maintenance of the MHB**

Gene expression analyses have previously shown that deletion of Fgfr1 disrupts the coherence of the MHB (Trokovic et al., 2005; Trokovic et al., 2003). However, whether the FGF pathway is required for cell sorting at the MHB has not yet been examined. To this end, we deleted Fgf8, the crucial Fgf ligand for Fgfr1 in mes-1 development, in a mosaic manner and simultaneously fate-mapped Gbx2-expressing cells in \( Fgf8^{+/-}, \ Gbx2^{\text{CreER}^{+/-}}, \ R26^{\text{RlacZ}^{+/-}} \) embryos by administering tamoxifen between E6.75 and E8.5. Remarkably, a large number of labeled descendants of Gbx2-expressing cells were found in the midbrain of \( Fgf8^{+/-}, \ Gbx2^{\text{CreER}^{+/-}}, \ R26^{\text{RlacZ}^{+/-}} \) embryos by E10.5 following tamoxifen administration at E6.75 \( (n=8, \text{Fig. 5E,F,I,K-L})\). In contrast to the uniform anterior shift of the Gbx2 lineage in Gbx2 mosaic deletion embryos, partial deletion of Fgf8 resulted in a broad haphazard distribution of labeled cells in the mes (compare Fig. 5D with 5F, and 5J with 5K). The fate-mapped cells in the mes expressed Otx2, suggesting a cell fate switch of these r1-derived cells (Fig. 5L').

Administration of tamoxifen at E8.5 resulted in a similar but less severe phenotype in \( Fgf8^{+/-}, \ Gbx2^{\text{CreER}^{+/-}}, \ R26^{\text{RlacZ}^{+/-}} \) embryos \( (n=6, \text{see Fig. S2C in the supplementary material})\). These results demonstrate that Fgf8 is required for the segregation of the mes and r1 in maintaining the lineage boundary at the MHB.

Next, we examined the effect of mosaic deletion of Fgf8 on the expression of MHB genes in \( Fgf8^{+/-}, \ Gbx2^{\text{CreER}^{+/-}}, \ R26^{\text{RlacZ}^{+/-}} \) embryos at E9.5 and E10.5 following tamoxifen administration at E6.5. In these mutant embryos at E9.5, Gbx2 expression was maintained in anterior r1, but its expression in the dorsalmost part of r1 was greatly reduced (Fig. 6C). To examine the deletion of Fgf8, we performed in situ hybridization using an RNA probe corresponding to Fgf8 exon 3, which is deleted by Cre-mediated recombination (Meyers et al., 1998). The intact Fgf8 transcripts were noticeably reduced in the isthmus and almost completely lost in the dorsal- and ventralmost parts of the isthmus in E9.5 \( Fgf8^{+/-}, \ Gbx2^{\text{CreER}^{+/-}}, \ R26^{\text{RlacZ}^{+/-}} \) embryos that were given tamoxifen at E6.75, confirming the mosaic deletion of Fgf8 in r1 (Fig. 6F and see Fig. S3 in the supplementary material). At E10.5, the transverse band of Fgf8 in the isthmus broadened and became irregular (inset in Fig. 6F). In these mutant embryos, the transverse stripe of Wnt1 expression at the MHB was initially present at E9.5 but disappeared at E10.5 (Fig. 6I). Similar to the progressive disruption of Fgf8 and Wnt1 expression, Otx2 was mostly normal at E9.5, but by E10.5, the causal border of Otx2 was highly irregular and shifted posteriorly (Fig. 6L and see Fig. S3 in the supplementary material). These observations show that the coherence of the MHB is progressively disrupted between E9.5 and E10.5 in \( Fgf8^{+/-}, \ Gbx2^{\text{CreER}^{+/-}}, \ R26^{\text{RlacZ}^{+/-}} \) embryos that receive tamoxifen at E6.5, demonstrating that Fgf8 is essential for the maintenance of the MHB.

**Activation of FGF signaling results in cell aggregation in the mes**

Fgf8 is normally restricted to a tight transverse ring of cells immediately posterior to the MHB, implying stronger Fgf8 signaling in cells near the border than those farther away from it. This differential Fgf8 activity may play a role in restricting cell movements across the MHB. To test this model, we examined whether activation of Fgf8 signaling affects cell sorting in the mes-1 area. It has been demonstrated that forced expression of a constitutively active human FGF receptor, FGFR1K656E, which contains a mutation in the tyrosine kinase domains, or Fgf8, results in an identical response in gene expression in chick embryos (Liu et al., 2003). We thus studied the behavior of cells transfected with FGFR1K656E in the neural tube of chick embryos using in ovo
Cells transfected with EGFP were evenly distributed throughout the mes-r1 region (Fig. 7A,C,G,G/H11033). By contrast, cells transfected with FGFR1K656E and EGFP segregated from untransfected cells in the mes (Fig. 7B,D,H,H/H11033). The aggregates of transfected cells displayed smooth borders, and abnormal folding of the neuroepithelium was often associated with the cell segregation (Fig. 7D,H,H/H11033). Interestingly, forced expression of FGFR1K656E had no effect on cell sorting in r1 (Fig. 7D,H/H11032,H/H11033).

Electroporation of two other constitutively active FGFRs, FGFR1N546K or FGFR2C342Y (Liu et al., 2003), resulted in similar cell sorting phenotype in the mes (data not shown). Therefore, activation of FGF signaling has a cell-autonomous effect on cell sorting in the mes.

It has been shown that Fgf8 signaling negatively regulates Otx2 (Liu et al., 1999; Martinez et al., 1999). Indeed, double immunofluorescence analysis showed that Otx2 expression was inhibited in cells transfected with FGFR1K656E and EGFP segregated from untransfected cells in the mes (Fig. 7B,D,H,H'). The aggregates of transfected cells displayed smooth borders, and abnormal folding of the neuroepithelium was often associated with the cell segregation (Fig. 7D,H,H'). Interestingly, forced expression of FGFR1K656E had no effect on cell sorting in r1 (Fig. 7D,H',H').

Electroporation of two other constitutively active FGFRs, FGFR1N546K or FGFR2C342Y (Liu et al., 2003), resulted in similar cell sorting phenotype in the mes (data not shown). Therefore, activation of FGF signaling has a cell-autonomous effect on cell sorting in the mes.

It has been shown that Fgf8 signaling negatively regulates Otx2 (Liu et al., 1999; Martinez et al., 1999). Indeed, double immunofluorescence analysis showed that Otx2 expression was inhibited in cells transfected with FGFR1K656E but was unaffected by transfection of EGFP in the mes (Fig. 7G-H'). To test whether FGF-induced cell sorting is attributable to the loss of Otx2 in the mes, we examined the behavior of cells transfected with Otx2 alone, or FGFR1K656E and Otx2 together. As expected, forced expression of Otx2 had no effect in the mes (Fig. 7E,I,I'). However, forced expression of Otx2 alone, or Otx2 and FGFR1K656E resulted in cell segregation in r1 (Fig. 7E,F,K-K/L-L/H11033), demonstrating that ectopic expression of Otx2 induces cell segregation in r1. Importantly, although Otx2 expression persisted in transfected cells, forced expression of both Otx2 and FGFR1K656E caused cell segregation and abnormal folding of the neuroepithelium in the mes similar to that found in embryos transfected with FGFR1K656E alone (Fig. 7F,I-I'). To rule out the possibility that transfected cells may first lose the endogenous Otx2 and aggregate before the onset of exogenous Otx2 expression, we examined the expression of Otx2 in chick embryos transfected with Otx2 and FGFR1K656E 12 or 24 hours after electroporation. Ectopic expression of Otx2 was detected in r1 by 12 hours, whereas no inhibition of the endogenous Otx2 was found 12 or 24 hours after electroporation (see Fig. S4 in the supplementary material). These data collectively demonstrate that activation of FGF signaling pathways has a cell-autonomous function in regulating cell sorting independent of the repression of Otx2 in the mes.
Formation of the mid-hindbrain lineage boundary

**DISCUSSION**

Previous studies have demonstrated that mutual inhibition between **Otx2** and **Gbx2** determined the position of the prospective MHB in the neural plate at E7.5 (Broccoli et al., 1999; Li and Joyner, 2001; Millet et al., 1999). Here, we demonstrate that **Gbx2** specifies r1 fate, and **Gbx2**-expressing r1 cells and their progeny are mostly restricted from crossing the MHB boundary between E7.5 and E14.5. Therefore, reciprocal inhibition between **Otx2** and **Gbx2** results in their differential expression in mes and r1 progenitor cells, which in turn leads to the initial segregation of mes and r1 cells. Furthermore, we show that **Fgf8** activity is important for the maintenance of the MHB by regulating cell sorting. Our results demonstrate the temporal requirements for **Gbx2** and **Fgf8** in the development of the compartment boundary at the compartment boundary at the MHB.

**The rostral expression border of Gbx2 defines the prospective lineage boundary at the MHB in the neural plate**

In mouse embryos, **Gbx2** is expressed throughout the posterior part of the embryo, with the anterior limit abutting the expression domain of **Otx2** as early as E7.5. The juxtaposition of **Gbx2** and **Otx2** expression is maintained as late as E14.5 (Li and Joyner, 2001; Li et al., 2002). Using G1Fm with the **Gbx2**^CreER^ allele, we showed that r1 cells, once they express **Gbx2**, are restricted from entering the mes in mouse embryos at least until E14.5. Therefore, the MHB boundary is coupled with the heritable expression of **Gbx2** in r1 cells. Our findings are in agreement with a previous report that suggested that lineage restriction is probably established at the MHB at the end of gastrulation in zebrafish (Langenberg and Brand, 2005).

A G1Fm study using a **Wnt1**-**CreER** transgenic mouse line to label mes cells suggests that lineage restriction may not occur in either the alar plate until E8.5, or in the basal plate until E9.5 (Zervas et al., 2004). There are several possible explanations for the apparent discrepancy between the G1Fm studies using the **Wnt1**-**CreER** and **Gbx2**^CreER^ alleles. First, the initial expression of **Wnt1** may be present in some **Gbx2**^+^ cells before E8.5. Second, it is possible that the **Wnt1**-**CreER** transgene does not completely recapitulate the endogenous **Wnt1** expression so that there may be ectopic expression of **CreER** in r1 progenitors between E8.5 and E9.5. Finally, some mes-derived cells may migrate across the MHB at early somite stages. Indeed, we found streams of postmitotic **Otx2**^+^ neural precursors present in anterior r1 at E9.5 and E10.5 (Fig. 1E-F,H-J). We found that these **Otx2**^+^ cells in anterior r1 were not derived from the **Gbx2**-lineage, demonstrating that some mes cells may enter r1 after they exit the cell cycle (data not shown). These mes-derived cells may represent the descendants of **Wnt1**-expressing cells that were previously found to violate the MHB between E8.5 and E9.5 (Zervas et al., 2004). Interestingly, we found that some **Gbx2**-derived cells entered the mes as postmitotic neuronal precursors after E14.5 (K. Kala, J.Y.H.L., M. Salminem and J. Partanen, unpublished). Therefore, the lineage-restriction boundary at the MHB may primarily apply to the proliferating progenitors. Movements of postmitotic cells across the compartment boundary after they are specified may represent an important mechanism in generating greater cellular diversity in the nervous system (Kiecker and Lumsden, 2005).

**The sorting of Gbx2+ hindbrain and Otx2+ midbrain cells leads to the establishment of the MHB boundary**

Using chimera and chick electroporation analyses, we showed that ectopic expression of **Otx2** causes cell segregation in r1 (Fig. 4E-H and Fig. 7E). In a similar manner, **Otx2**-deficient cells segregate from wild-type cells in the mes of mouse chimeras composed of wild-type and mutant cells or in chick embryos (Rhinn et al., 1999) (Fig. 7D,H-I). These results strongly suggest that differential expression of **Otx2** and **Gbx2** in mes and r1 cells, respectively, contributes to the segregation of these two cell populations during the establishment of the MHB. Therefore, the MHB is a lineage-restriction boundary coupled with a sorting mechanism.

We have previously demonstrated that induction of **CreER** by tamoxifen results in simultaneously mosaic ‘self-deletion’ of **Gbx2** and fate mapping the **Gbx2** lineage in **Gbx2**^CreER; R26R**^{loxZ/lox} embryos (Chen, L. et al., 2009). As the efficiency of **CreER**-mediated recombination may be different at the **Gbx2** and **R26R** loci, we cannot be certain that **Gbx2** is deleted in individual β-gal^+^ cells. However, the expression of β-gal is probably associated with the inactivation of **Gbx2** in general. Indeed, abnormal expression of **Wnt1** and **Fgf8** was found in r1 of **Gbx2**^CreER; R26R**^{loxZ/lox} embryos by E9.5, and the similar misregulation of **Wnt1** and **Fgf8** has been associated with the loss of **Gbx2** or ectopic expression of **Otx2** in r1 (Li et al., 2002; Ye et al., 2001). In these **Gbx2** mosaic
mutant embryos, the anterior domain of the fate-mapped Gbx2 lineage overlapped with Otx2 expression, suggesting that r1 cells that lose Gbx2 near the MHB sort into the mes. Interestingly, a relatively intact MHB was found posterior to the mutant or the fate-mapped cells, suggesting that the Gbx2 lineage border and cell segregation border no longer coincides in these mutants. In agreement with our previous finding (Li et al., 2002), deletion of Gbx2 after E8.5 had little effect on the MHB, demonstrating that a Gbx2-independent pathway is required to maintain cell segregation at the MHB (see Fig. S2B in the supplementary material). We showed that deletion of Gbx2 at E7.5 resulted in upregulation of Fgf8 expression in r1 (Fig. 6E). The elevated Fgf8 expression may not only compensate for the loss of Gbx2 in repressing Otx2 from r1 cells as suggested previously (Li et al., 2002), but may also play an important role in establishing a new cell segregation boundary posterior to the Gbx2-lineage border as discussed below.

**Fgf8 plays a crucial role in the maintenance of the lineage boundary at the MHB**

The progeny of Gbx2-expressing cells were found broadly in the mes following mosaic deletion of Fgf8 after E7.0 in Fgf8<sup>F/L</sup>; Gbx2<sup>CreER<sup>+</sup></sup>; R26RlacZ/+ embryos. This phenotype may result from disruption of lineage restriction or ectopic expression of Gbx2 (CreER) in the mes. Although we cannot completely rule out the latter, several observations support the argument against this explanation. First of all, CreER-mediated deletion of Fgf8 in r1 cells by administration of tamoxifen at E6.75 probably occurs before or during the onset of Fgf8 expression, which is initiated in anterior r1 between E8.0 and E8.5 (Liu and Joyner, 2001). However, the disruption of the MHB boundary mainly occurred after E9.5 in Fgf8<sup>F/L</sup>; Gbx2<sup>CreER<sup>+</sup></sup>; R26RlacZ/+ embryos that were given tamoxifen at E6.75 (Fig. 6 and see Fig. S3 in the supplementary material). Furthermore, given the 6 to 36 hour
window of activation by a single dose of tamoxifen, CreER, if it is ectopically induced in the mes owing to partial loss of Fgf8, is unlikely to be active by E9.5. Finally, deletion of Fgf8 after E8.5 resulted in similar disruption of the lineage restriction at the MHB (see Fig. S2C in the supplementary material). Therefore, our results extend the previous findings that FGF signaling is essential for maintaining the MHB defined by gene expression, by demonstrating that Fgf8 is required for maintaining the lineage restriction at the MHB (Trokovik et al., 2005).

How does Fgf8 signaling maintain the MHB boundary? In Fgf8/−; Gbx2CreER+/−; R26RlacZ/+ embryos that were given tamoxifen at E6.75, Gbx2-derived cells expressed Otx2 and intermingled with mes cells, demonstrating that these r1-derived cells had undergone cell fate respecification (Fig. 5L-L' and see Fig. S3 in the supplementary material). Indeed, ectopic Otx2+ cells had undergone cell fate respecification (Fig. 5L-L' and see Fig. S3 in the supplementary material). Therefore, Fgf8 may regulate cell sorting by maintaining r1 fate. However, the following observations indicated that Fgf8 signaling probably has a more direct role in restricting cell movements at the MHB. In contrast to the restricted distribution of r1-derived cells in Gbx2 mosaic deletion embryos, cells originating from r1 were scattered in a much broader domain of the mes when Fgf8 was deleted in a mosaic manner (Fig. 5C-F). Furthermore, activation of FGF signaling has a cell-autonomous role in controlling cell sorting in the mes. Finally, previous studies have shown that Fgf1 is required for the expression of Cdh22, which encodes the cell adhesion molecule PB-cadherin, in the posterior mes (Trokovik et al., 2003), and that FGF signaling modulates ephrin and catenins in regulating cell-cell interactions (Lee et al., 2009; Lee et al., 2008; Lilien et al., 2002). As Fgf8-expressing cells form a tight transverse band in the isthmus, the mes cells immediately anterior to the MHB probably receive high levels of Fgf8 and thus display distinct cell adhesive characteristics. Significantly, forced expression of FGFR1K656E did not cause cell aggregation in r1, demonstrating that activation of FGF does not alter cell adhesion in r1 (Fig. 7D,H). Therefore, the different responses between mes and r1 cells to FGF signaling suggest that there is an abrupt change in cell adhesion at the MHB, preventing cell movements across the MHB. However, within the mes compartment, where the difference in cell adhesion modulated by Fgf8 signaling is gradual and inductive, cells can intermingled. Future studies will try to determine why FGF signaling regulates cellular sorting in the mes but not r1, and what are the effector molecules that mediate the FGF pathway in regulating cell sorting.

**Evolutionarily conserved principles in development of compartment boundaries**

The stepwise formation of the compartment boundary at the MHB regulated by Gbx2 and Fgf8 is remarkably analogous to the development of the anteroposterior (AP) compartment boundary in the wing imaginal disc of *Drosophila*. In the wing disc, cells in the posterior compartment express the selector gene en grapheal (en) (Dahmann and Basler, 2009; Martin et al., 2009). Following the establishment of the boundary, the signaling molecule Hedgehog (Hh) is produced in the posterior cells near the boundary and it plays an important role in stabilizing the AP compartment boundary by a unidirectional action on anterior cells near the border (Blair and Ralston, 1997; Rodriguez and Basler, 1997). Interestingly, in the *Drosophila* wing disc, Hh acts only in a short range and induces decapenmatapeligic (DPP), a member of the transforming growth factor β superfamily, in a row of anterior cells along the compartment boundary (Dahmann and Basler, 1999). DPP in turn acts as a long-range morphogen in controlling the growth and patterning of the anterior and posterior compartments (Dahmann and Basler, 1999). Different from Hh, Fgf8 is known to diffuse away from the producing cells, forming a morphogen gradient to pattern the mes-r1 area (Chen, Y., et al., 2009; Schoell and Brand, 2004; Yu et al., 2009). Therefore, Fgf8 appears to have dual roles in stabilizing the compartment boundary at the MHB and exerting long-range effects in patterning the developing midbrain and hindbrain.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

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**References**


