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

N. Abimbola Sunmonu*, Kairong Li*, Qiuxia Guo and James Y. H. Li†

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 *Gbx2*^{CreER} 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 (Trokovic et al., 2005; Trokovic 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.

Department of Genetics and Developmental Biology, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030, USA.

*These authors contributed equally to this work
†Author for correspondence (jali@uchc.edu)

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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 *Fgf8* between E7.5 and E9.5. By combining GIFM, chimeric and genetic mosaic studies, we have uncovered sequential roles of *Gbx2* and *Fgf8* 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 *Fgf8* 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: $Gbx2^{F}$ (Li et al., 2002); $Fgf8^{-}$ and $Fgf8^{F}$ (Sun et al., 2002); $R26R^{lacZ}$ and $R26R^{YFP}$ (Soriano, 1999). CreER-mediated recombination in $Gbx2^{CreER/F}$; $R26R^{lacZ/+}$ or $Fgf8^{F/-}$; $Gbx2^{CreER/+}$; $R26R^{lacZ/+}$ embryos that received tamoxifen between E6.5 and E8.5 was determined by 5-bromo-4-chloro-3-indolyl-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://lilab.uchc.edu/Pages/Protocols.html). The following antibodies were used: rabbit anti-GFP IgG fraction (1:1,000, Invitrogen, Carisbad, CA, USA), goat-anti-Otx2 (1:150, R&D system, Minneapolis, MN, USA), and mouse anti-TuJ1 (Covance).

Chick electroporation studies

Full-length cDNAs for $FGFR1^{K656E}$, $FGFR1^{N546K}$, or $FGFR2^{C342Y}$ were cloned into the expression vector *pMiwIII* (Olsen et al., 2006). *Otx2* was cloned into a modified *pMiwIII*, *pMiwIII-ires-EGFP*, so that Otx2 and EGFP were co-expressed in the transfected cells. Chick electroporation assays were performed as described previously (Liu et al., 2003). Embryos were dissected after 30 or 48 hours and processed for immunohistochemistry. For each construct, at least five transfected embryos were analyzed, and consistent phenotypes were observed for each construct.

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, $R26R^{lacZ}$ (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.

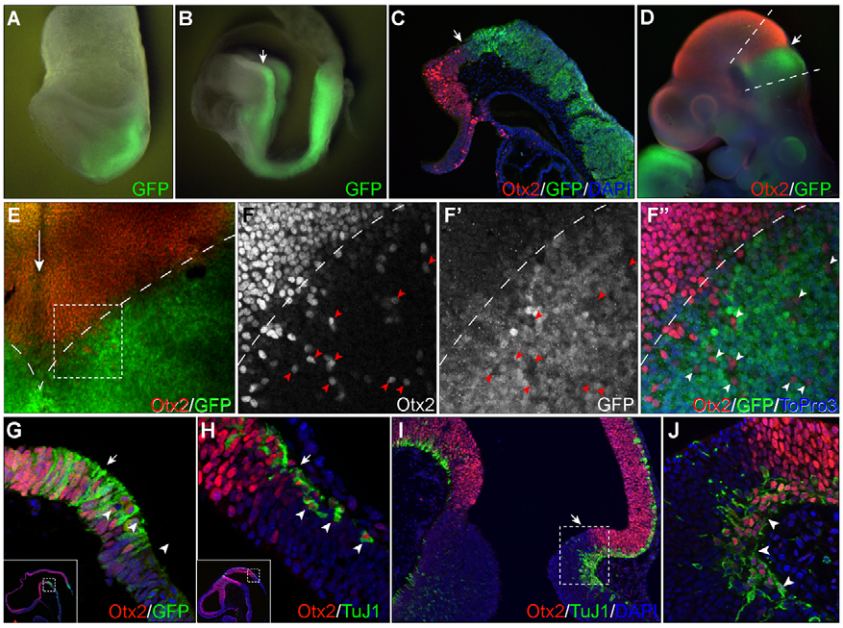

Fig. 1. Analysis of *Otx2* and *EGFP* expression, which mimics endogenous *Gbx2*, in the mes–r1 area of *Gbx2*$^{CreER/+}$ embryos. (**A**,**B**) EGFP fluorescence in *Gbx2*$^{CreER/+}$ embryos at E7.5 (A) and E8.5 (B). (**C**,**D**) Immunofluorescence of Otx2 and GFP on sagittal sections of *Gbx2*$^{CreER/+}$ embryos at E8.5 (C) and whole-mount embryos at E9.5 (D). Arrows mark the prospective MHB. (**E-F″**) Confocal images of Otx2/GFP immunofluorescence in flat-mount E9.5 neural tube segment indicated by dashed lines in D. The boxed area in E is magnified in F–F″. The MHB is marked by a dashed line; the arrow indicates the ventral midline; arrowheads mark Otx2^{+}/GFP^{-} cells in r1 region. (**G**) Immunofluorescence of Otx2 and GFP on sagittal sections of *Gbx2*$^{CreER/+}$ embryos at E9.5 showing overlapping expression of EGFP and Otx2 in the ventral midline of the mes. The inset shows the relative position (boxed area) of G. (**H-J**) Immunofluorescence of Otx2 and TuJ1 on sagittal sections of *Gbx2*$^{CreER/+}$ embryos at E9.5 (H) and E10.5 (I,J). The inset shows the relative position of H in the dorsal mes-r1 junction (box). Otx2^{+} cells (arrowheads) in anterior r1 express TuJ1. The boxed area in I is enlarged in J. The arrowhead indicates Otx2^{+} cells in anterior 1.

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 *Gbx2*$^{CreER/-}$; *R26R*$^{lacZ/+}$ embryos. The cerebellar primordium was absent in *Gbx2*$^{CreER/-}$; *R26R*$^{lacZ/+}$ embryos as found in *Gbx2*$^{-/-}$ embryos, demonstrating that *Gbx2*^{CreER} is a null allele (Fig. 3B,D). Significantly, descendants of *Gbx2*-transcribing cells were found throughout the midbrain in *Gbx2*$^{CreER/-}$; *R26R*$^{lacZ/+}$ embryos (Fig. 3B,D). Moreover, the marked cells expressed Otx2 and intermixed with midbrain cells in *Gbx2*$^{CreER/-}$; *R26R*$^{YFP/+}$ 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.

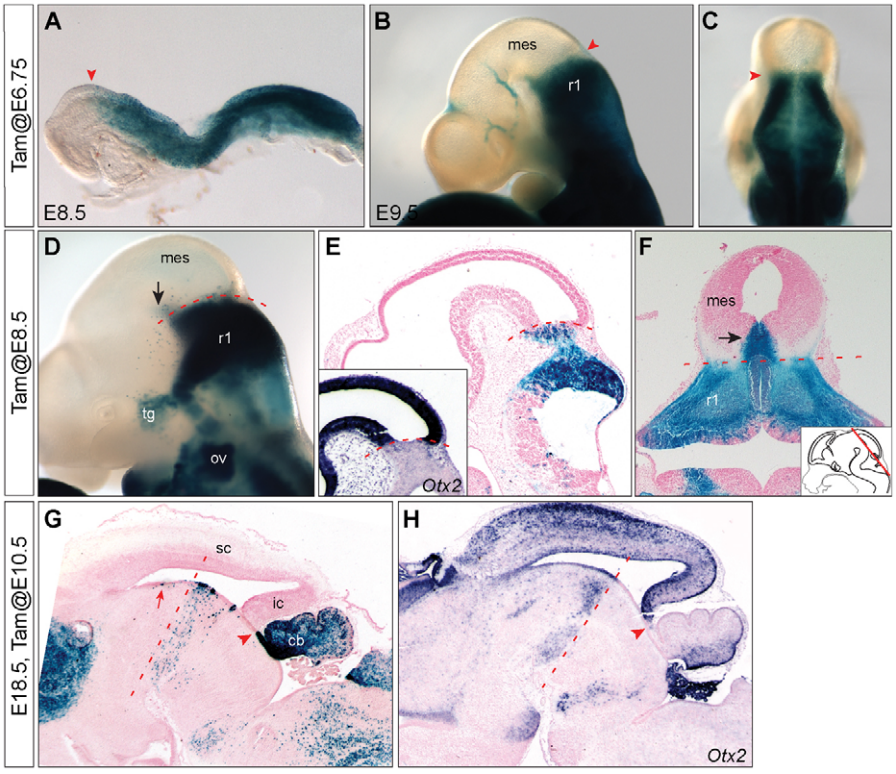

Fig. 2. Descendants of *Gbx2*-expressing cells are restricted posterior to the MHB. (**A-C**) X-gal histochemistry of *Gbx2*$^{CreER/+}$; *R26R*$^{lacZ/+}$ embryos at E8.5 (A) and E9.5 (B,C) following tamoxifen administration at E6.75. The arrowhead indicates the MHB. (**D-F**) X-gal histochemistry of whole-mount (D) and sagittal section of E10.5 *Gbx2*$^{CreER/+}$; *R26R*$^{lacZ/+}$ 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 *Gbx2*$^{CreER/+}$; *R26R*$^{lacZ/+}$ embryos at E18.5 after tamoxifen gavage at E10.5. cb, cerebellum; ic, inferior colliculus; ov, otic vesicle; sc, superior colliculus; tg, trigeminal ganglion.

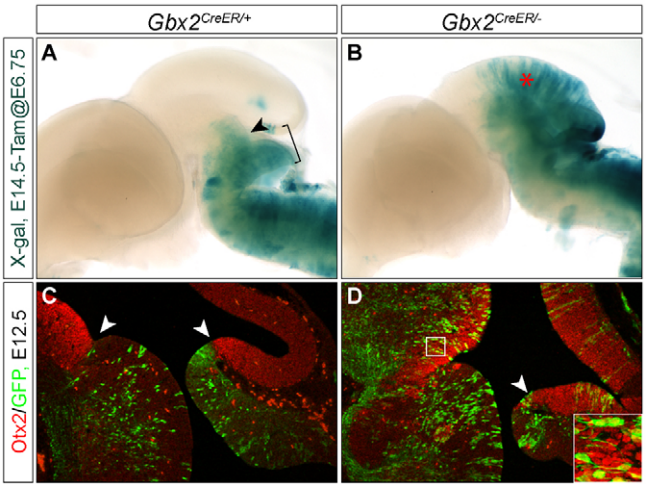

Fig. 3. Descendants of *Gbx2*-transcribing cells contribute to the midbrain in *Gbx2*-deficient mice. (**A**,**B**) X-gal histochemistry of whole-mount *Gbx2*$^{CreER/+}$; *R26R*$^{lacZ/+}$ (A) and *Gbx2*$^{CreER/–}$; *R26R*$^{lacZ/+}$ embryos (B) at E14.5 after tamoxifen administration at E6.75. (**C**,**D**) Immunofluorescence of Otx2 and YFP on sagittal sections of E12.5 *Gbx2*$^{CreER/+}$; *R26R*$^{YFP/+}$ (C) and *Gbx2*$^{CreER/–}$; *R26R*$^{YFP/+}$ embryos (D) given tamoxifen at E6.5. Note that the marked cells (asterisk) are present throughout the midbrain in *Gbx2*-deficient embryos, in contrast to the lineage restriction at the MHB (arrowhead) in the control embryos. The inset in D shows that the fate-mapped cells (YFP$^+$) express Otx2. The bracket marks r1.

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 *Gbx2*^{CreER} allele with a conditional mutant allele, *Gbx2*^{Floxed} (*Gbx2*^{F}) and administering tamoxifen to *Gbx2*$^{CreER/F}$; *R26R*$^{lacZ/+}$ 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 *Gbx2*$^{CreER/F}$ (phenotypically normal) to *Gbx2*$^{CreER/–}$ (*Gbx2* deficient). The recombined cells have a high probability of being marked by β-gal in *Gbx2*$^{CreER/F}$; *R26R*$^{lacZ/+}$ mice.

In *Gbx2*$^{CreER/F}$; *R26R*$^{lacZ/+}$ 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 *Gbx2*$^{CreER/F}$; *R26R*$^{lacZ/+}$ embryos, suggesting mosaic deletion of *Gbx2* in r1 cells (Fig. 5H inset and Fig. 5J). Although the labeled r1 cells formed a clear anterior border, this border had apparently shifted rostrally in *Gbx2*$^{CreER/F}$; *R26R*$^{lacZ/+}$ 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 *Gbx2*$^{CreER/F}$; *R26R*$^{lacZ/YFP}$ embryos that were given tamoxifen at E6.75. Many labeled cells were found in the posterior part of the mes and expressed Otx2 (Fig. 5J). 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 *Gbx2*$^{CreER/F}$; *R26R*$^{lacZ/–}$ 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 *Gbx2*$^{CreER/F}$; *R26R*$^{lacZ/+}$ 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 *Gbx2*$^{CreER/F}$; *R26R*$^{lacZ/+}$ 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 *Gbx2*$^{CreER/F}$; *R26R*$^{lacZ/+}$ 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 *Gbx2*$^{CreER/F}$; *R26R*$^{lacZ/–}$ embryos at E8.5. No

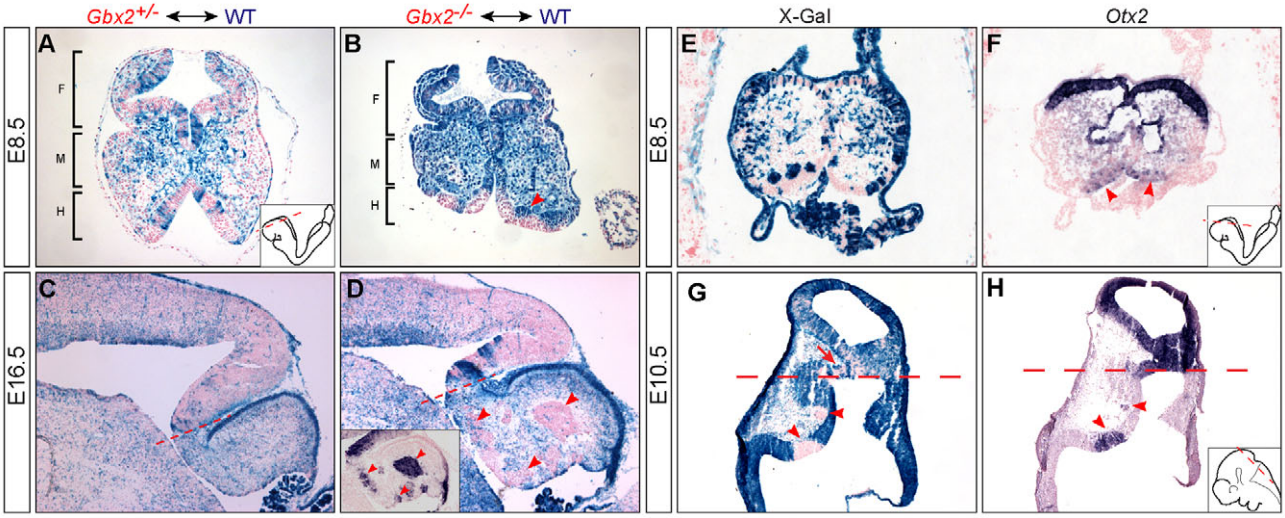

Fig. 4. *Gbx2*-deficient cells ectopically express *Otx2* and segregate from wild-type cells in the anterior hindbrain of chimeric embryos. (**A-D**) X-gal histochemistry of sections of chimeric embryos at E8.5 (A and B) and E16.5 (C and D). The inset in D is in situ hybridization of *Otx2* transcripts on an adjacent section. (**E-H**) X-gal and in situ hybridization on adjacent horizontal sections of mutant chimeric embryos at the five-somite stage (E,F) and E10.5 (G,H). The planes of section are illustrated in the insets. The arrowheads indicate aggregates composed of either wild-type or mutant cells in r1; the arrow indicates the accumulation of mutant cells in the posterior mes; the dashed line marks the MHB boundary. F, forebrain; H, hindbrain; M, midbrain.

significant defects were detected at the MHB (n=9, 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 at 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-r1 development, in a mosaic manner and simultaneously fate-mapped *Gbx2*-expressing cells in $Fgf8^{F/-}$; $Gbx2^{CreER/+}$; $R26R^{lacZ/+}$ 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 $Fgf8^{F/-}$; $Gbx2^{CreER/+}$; $R26R^{lacZ/+}$ embryos by E10.5 following tamoxifen administration at E6.75 (n=8, 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 $Fgf8^{F/F}$; $Gbx2^{CreER/+}$; $R26R^{lacZ/+}$ embryos (n=6, 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 $Fgf8^{F/-}$; $Gbx2^{CreER/+}$; $R26R^{lacZ/+}$ 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 $Fgf8^{F/-}$; $Gbx2^{CreER/+}$; $R26R^{lacZ/+}$ 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 caudal 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 $Fgf8^{F/-}$; $Gbx2^{CreER/+}$; $R26R^{lacZ/+}$ 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-r1 area. It has been demonstrated that forced expression of a constitutively active human FGF receptor, $FGFR1^{K656E}$, 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 $FGFR1^{K656E}$ in the neural tube of chick embryos using in ovo

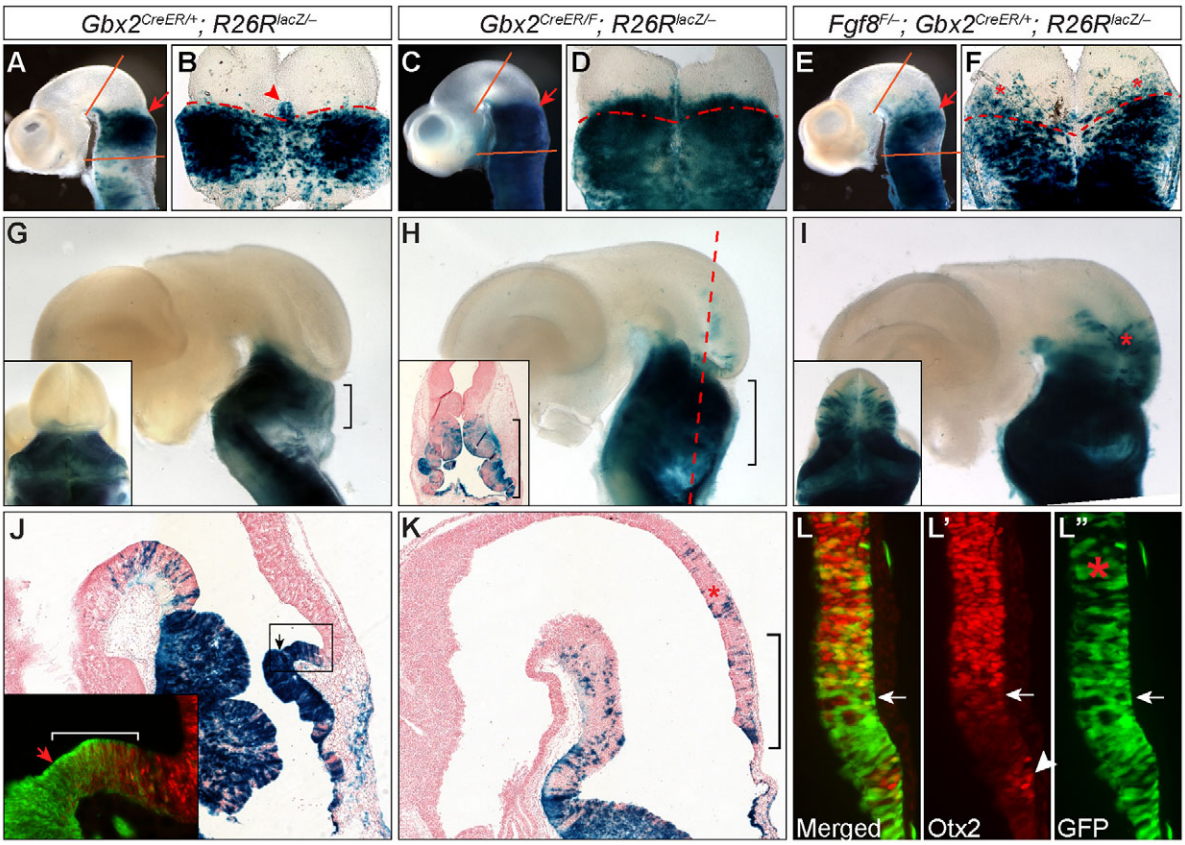

Fig. 5. Different requirements for *Fgf8* and *Gbx2* in the maintenance of the lineage boundary at the MHB. (**A-I**) X-gal analysis of whole-mount (A,C,E), flat-mount (B,D,F) E10.5 brains, and whole-mount (G,H,I) E12.5 brains of indicated genotypes. Tamoxifen was given at E6.75. The neural tube segment used for flat-mount preparations is demarcated with red lines. The dashed line and arrows mark the isthmus. Insets show the dorsal view of the embryos in G and I, and the coronal section of the embryo in H. The dashed line in H indicates the plane of section. Note that the neuroepithelium corresponding to r1 is enlarged (bracket) and abnormally folded. (**J**,**K**) X-gal histochemistry on sagittal section of *Gbx2*$^{CreER/F}$; *R26R*$^{YFP/lacZ}$ (J) and *Gbx2*$^{CreER/+}$; *Fgf8*$^{F/–}$; *R26R*$^{lacZ/+}$ (K) embryos at E12.5 following tamoxifen exposure at E6.75. The inset in J shows double immunofluorescence of Otx2 and GFP on the adjacent section of J. Note that the anterior border of the fate-mapped cells is in the posterior mes (J), and that the cerebellar primordium was noticeably smaller, caused by the mosaic deletion of *Fgf8* (K). (**L-L″**) Double immunofluorescence for Otx2 and GFP on the sagittal section of E10.5 *Gbx2*$^{CreER/+}$; *Fgf82*$^{F/–}$; *R26R*$^{YFP/+}$ that received tamoxifen at E6.75. The image corresponds to the dorsal part of the MHB, as indicated by the bracket in K. The asterisk indicates the fate-mapped cells in the mes.

electroporation. Cells transfected with *EGFP* were evenly distributed throughout the mes-r1 region (Fig. 7A,C,G,G″). By contrast, cells transfected with *FGFR1*^{K656E} 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 *FGFR*^{IK656E} had no effect on cell sorting in r1 (Fig. 7D,H′,H″). Electroporation of two other constitutively active *FGFRs*, *FGFR1*^{N546K} or *FGFR2*^{C342Y} (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 *FGFR*^{IK656E} 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 *FGFR1*^{K656E} 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 *FGFR1*^{K656E} resulted in cell segregation in r1 (Fig. 7E,F,K-K″,L-L″), 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 *FGFR1*^{K656E} caused cell segregation and abnormal folding of the neuroepithelium in the mes similar to that found in embryos transfected with *FGFR1*^{K656E} 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 *FGFR1*^{K656E} 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.

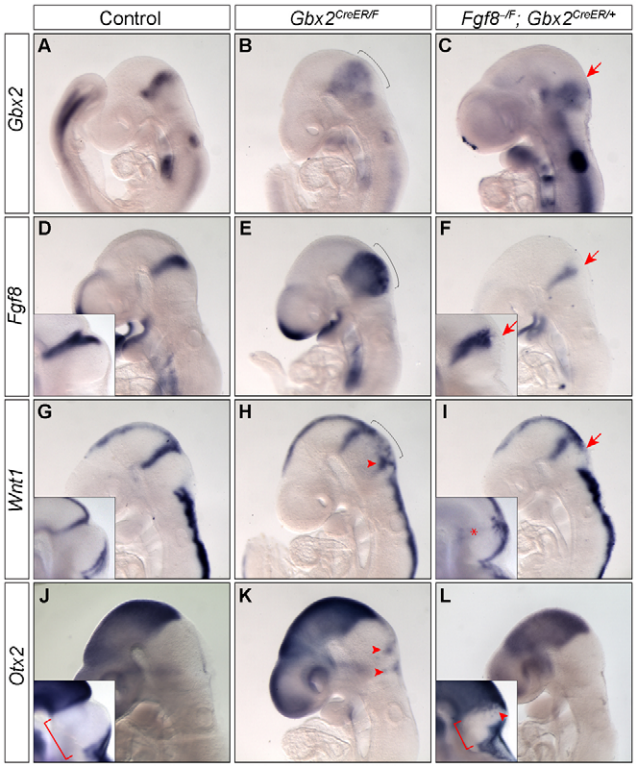

Fig. 6. Mosaic deletion of *Gbx2* or *Fgf8* results in distinct responses in gene expression in the mes-r1 area. (**A-L**) In situ hybridization of whole-mount embryos at E9.5 and E10.5 (insets). Genotypes and probes are indicated at the top and left, respectively. Arrowheads indicate ectopic expression in patches of r1 cells; arrows indicate the loss of expression of *Fgf8* and *Gbx2* or the ectopic expression of *Wnt1* near the dorsal midline; black brackets in B, E and H mark the expanded expression domain of *Gbx2*, *Fgf8* and *Wnt1*, respectively; red brackets in the insets of J and L demarcate the r1 domain; the asterisk indicates the loss of the transverse band of *Wnt1* expression at the MHB.

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 GIFM 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 GIFM 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 GIFM 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-H″). 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 $Gbx2^{CreER/F}$; $R26R^{lacZ/+}$ 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 $Gbx2^{CreER/F}$; $R26R^{lacZ/+}$ 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

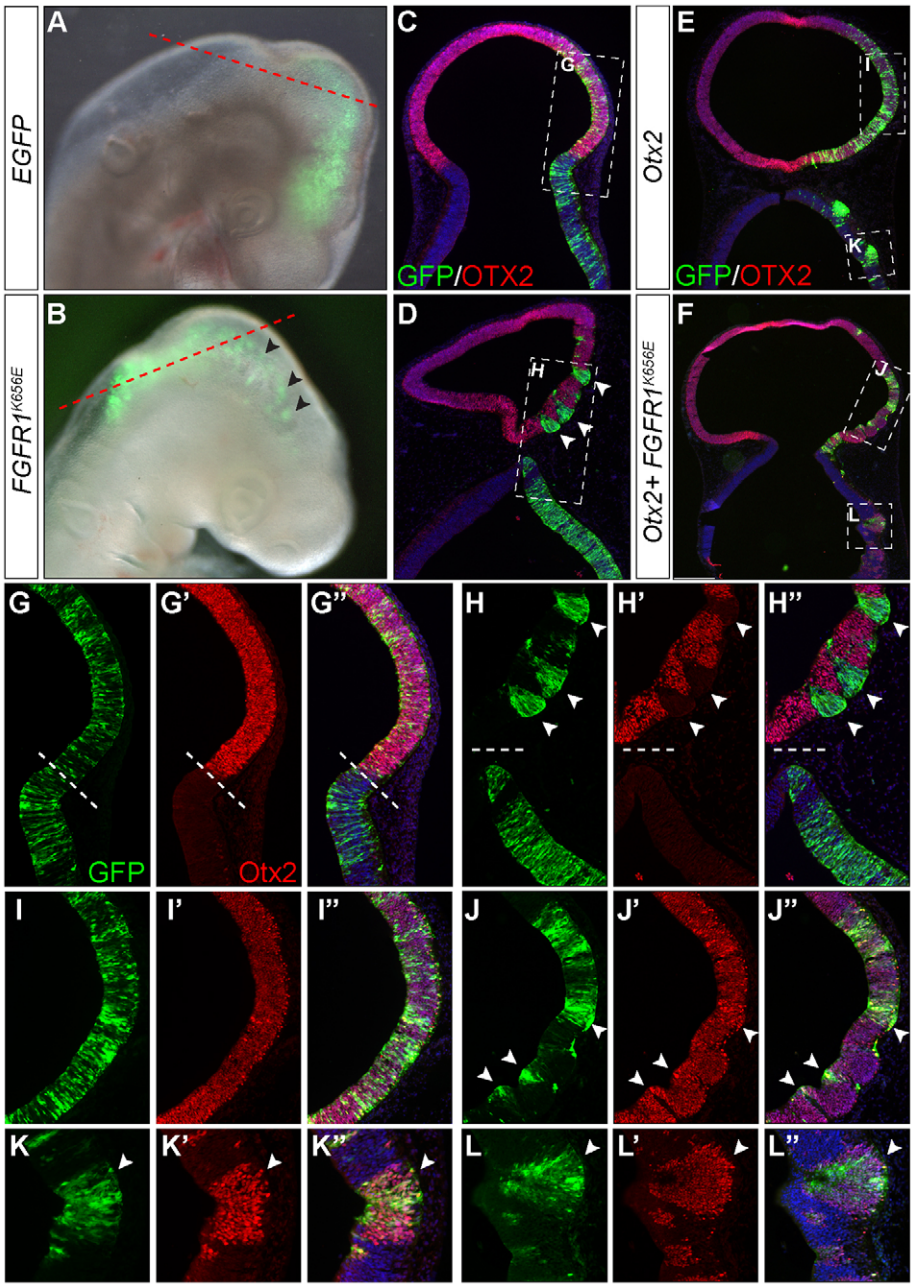

Fig. 7. Activation of FGF signaling has cell-autonomous function in cell sorting in the mes. (**A**,**B**) Distribution of cells transfected with EGFP alone (A) or EGFP with activated FGFR1 (B) in chick embryos at 48 hours after electroporation. (**C-L″**) Double fluorescence of GFP and Otx2 on horizontal sections of embryos transfected with EGFP (C,G-G″), activated FGFR1 (D and H-H″), Otx2 (E, I-I″,K-K″) and Otx2 with activated FGFR1 (F,J-J″,L-L″). Boxed areas in C, D, E and F are enlarged and shown in G-L″ as separate and merged color panels as indicated.

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 $Fgf8^{F/-}$; $Gbx2^{CreER/+}$; $R26R^{lacZ/+}$ 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 $Fgf8^{F/-}$; $Gbx2^{CreER/+}$; $R26R^{lacZ/+}$ 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 (Trokovic et al., 2005).

How does Fgf8 signaling maintain the MHB boundary? In $Fgf8^{F/-}$; $Gbx2^{CreER/+}$; $R26R^{lacZ/+}$ 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 were found in r1 of E10.5 $Fgf8^{F/-}$; $Gbx2^{CreER/+}$; $R26R^{lacZ/+}$ embryos that were given tamoxifen at E6.75 (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 *Fgfr1* is required for the expression of *Cdh22*, which encodes the cell adhesion molecule PB-cadherin, in the posterior mes (Trokovic 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 $FGFR1^{K656E}$ 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 intermingle. 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 *engrailed* (*en*), and the establishment of the AP compartment boundary is dependent on the heritable expression of *en* (Dahmann and Basler, 1999; 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 decapemataplegic (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; Scholpp 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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