New regulatory interactions and cellular responses in the isthmic organizer region revealed by altering Gbx2 expression

James Y. H. Li1,2,*, Zhimin Lao1 and Alexandra L. Joyner1,2,3

1Howard Hughes Medical Institute and Developmental Genetics Program, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, 540 First Avenue, New York, NY 10016, USA
2Department of Cell Biology, New York University School of Medicine, 540 First Avenue, New York, NY 10016, USA
3Department of Physiology and Neuroscience, New York University School of Medicine, 540 First Avenue, New York, NY 10016, USA

*Author for correspondence (e-mail: jali@uchc.edu)

Summary

The mouse homeobox gene Gbx2 is first expressed throughout the posterior region of the embryo during gastrulation, and becomes restricted to rhombomeres 1-3 (r1-3) by embryonic day 8.5 (E8.5). Previous studies have shown that r1-3 do not develop in Gbx2 mutants and that there is an early caudal expansion of the midbrain gene Otx2 to the anterior border of r4. Furthermore, expression of Wnt1 and Fgf8, two crucial components of the isthmic organizer, is no longer segregated to adjacent domains in Gbx2 mutants. In this study, we extend the phenotypic analysis of Gbx2 mutants by showing that Gbx2 is not only required for development of r1-3, but also for normal gene expression in r4-6. To determine whether Gbx2 can alter hindbrain development, we generated Hoxb1-Gbx2 (HG) transgenic mice in which Gbx2 is ectopically expressed in r4. We show that Gbx2 is not sufficient to induce r1-3 development in r4. To test whether an Otx2/Gbx2 interface can induce r1-3 development, we introduced the HG transgene onto a Gbx2-null mutant background and recreated a new Otx2/Gbx2 border in the anterior hindbrain. Development of r3, but not r1 and r2, is rescued in Gbx2−/−; HG embryos. In addition, the normal spatial relationship of Wnt1 and Fgf8 is established at the new Otx2/Gbx2 border, demonstrating that an interaction between Otx2 and Gbx2 is sufficient to produce the normal pattern of Wnt1 and Fgf8 expression. However, the expression domains of Fgf8 and Spry1, a downstream target of Fgf8, are greatly reduced in mid/hindbrain junction area of Gbx2−/−; HG embryos and the posterior midbrain is truncated because of abnormal cell death. Interestingly, we show that increased cell death and a partial loss of the midbrain are associated with increased expression of Fgf8 and Spry1 in Gbx2 conditional mutants that lack Gbx2 in r1 after E9.0. These results together suggest that cell survival in the posterior midbrain is positively or negatively regulated by Fgf8, depending on Fgf8 expression level. Our studies provide new insights into the regulatory interactions that maintain isthmic organizer gene expression and the consequences of altered levels of organizer gene expression on cell survival.

Key words: Gbx2, Mouse

Introduction

Development of the central nervous system (CNS) has provided an experimental paradigm with which to study the general process of pattern formation during embryogenesis. The complex adult CNS is derived from a seemingly homogenous neuroepithelium, which folds up to form a neural tube by embryonic day 9 (E9.0) in mouse. Based on morphology, the neural tube is partitioned into several domains, largely correlating with the prospective forebrain, midbrain, hindbrain and the spinal cord along the anteroposterior (AP) axis. These domains are further subdivided and refined by AP and dorsoventral (DV) positional information, which is determined by both intercellular and intracellular factors. One of the fundamental questions in developmental biology is how cells integrate intercellular and intracellular cues, and respond accordingly.

Acting as cell-intrinsic factors, homeodomain-containing proteins have been implicated in conferring positional values along the AP and DV axes of the CNS. In the caudal neural tube, Hox genes play an important role in establishing and maintaining positional identities of the hindbrain and the spinal cord (Lumsden and Krumlauf, 1996), whereas two homeobox genes, Otx2 and Gbx2, are crucial in regulating development of the rostral neural tube (Joyner et al., 2000; Wurst and Bally-Cuif, 2001). Otx2 is expressed in the anterior third of the mouse embryo at E7.5, and is maintained in the forebrain and midbrain at later stages (Ang et al., 1994). Correlating with the later expression, deletion of Otx2 in the neuroepithelium leads to a loss of the forebrain and the midbrain (Acampora et al., 1998; Rhinn et al., 1998). Complimentary to Otx2, the expression domain of Gbx2 extends from the posterior end of the embryo to the posterior limit of Otx2 at E7.5 (Bouillet et al., 1995; Wassarman et al., 1997). Gbx2 expression in the neuroepithelium is rapidly downregulated posterior to r3 after E7.5, and by E8.5 Gbx2 is strongly expressed in r1, and weakly in r2-3. Brain structures derived from rhombomere 1-3 (r1-3), including the cerebellum, fail to develop in Gbx2-null mouse
mutants (Wassarman et al., 1997), and Otx2 expression appears to be extended to the anterior limit of r4 by E8.5 (Millett et al., 1999). Removal of Otx2 rescues r3 development in Gbx2-null mutants, demonstrating that Gbx2 plays a permissive role in r3 development by repressing Otx2 (Li and Joyner, 2001). Conditional mutagenesis of Gbx2 further demonstrates that the repression of Otx2 by Gbx2 is required before E9.0 to allow development of r1-3 (Li et al., 2002). Although the genetic evidence has clearly demonstrated an essential role of Gbx2 in the development of r1-3 (Li et al., 2002). Although the genetic evidence has clearly demonstrated an essential role of Gbx2, it remains unknown whether Gbx2 is sufficient to specify cell fates in r1.

Acting in concert with cell-intrinsic factors, extrinsic factors are crucial in governing regionalization of the CNS. Embryological and genetic studies have demonstrated that there is a signaling center (the isthmic organizer) at the mid/hindbrain junction (isthmus) that plays a central role in patterning the developing midbrain and cerebellum. Two secreted factors, Wnt1 and Fgf8, are expressed at the mid/hindbrain junction, and deletion of Wnt1 or Fgf8 abolishes activity of the isthmic organizer leading to a loss of all midbrain and r1-derived structures (Chi et al., 2003; McMahon and Bradley, 1990; Meyers et al., 1998). Wnt1 and Fgf8 are normally expressed in two juxtaposed narrow domains at the Otx2/Gbx2 border with Wnt1 in the posterior Otx2 expression domain and Fgf8 in the anterior Gbx2 expression domain. This highly defined spatial expression pattern of Wnt1 and Fgf8 is dependent on Otx2 and Gbx2, because in mouse embryos that lack Gbx2 or both Otx2 and Gbx2, Wnt1 and Fgf8 are expressed in a broad overlapping domain (Li and Joyner, 2001; Martinez-Barbera et al., 2001; Millet et al., 1999; Wassarman et al., 1997). The distinct spatial expression patterns of Wnt1 and Fgf8 have been frequently used as an indicator for the normal formation of the isthmic organizer. However, the regulation and the biological significance of this spatial expression pattern are not fully understood.

Gain-of-function studies have demonstrated the remarkable inductive activity of Fgf8 in mimicking the activity of the isthmic organizer (Crossley et al., 1996; Liu et al., 2003; Martinez et al., 1999; Sato et al., 2001). Transplantation of beads soaked with Fgf8 recombinant protein in posterior forebrain or anterior midbrain induce midbrain or cerebellum tissue (Crossley et al., 1996; Martinez et al., 1999; Shamim et al., 1999). However, expression of Fgf8 in r4 is not able to transform r4 into a cerebellum in Gbx2-null mutant embryos (Millet et al., 1999), although an isthmus graft is capable of inducing an ectopic cerebellum in the posterior hindbrain, including r4 (Martinez et al., 1995). These observations raise the issue of whether a factor(s), possibly Gbx2, is missing that is required to mediate Fgf8 signaling to induce a cerebellum in r4 of Gbx2 mutants.

To investigate the active role of Gbx2 in specifying r1, we ectopically expressed Gbx2 in r4 using a mouse Hoxb1 enhancer. We show that Gbx2 is not sufficient to induce r1 genes in r4 in the transgenic mice (HG transgenics). As the Otx2 expression domain expands posteriorly at the late headfold stage in Gbx2-null mutants, we examined whether a new Otx2/Gbx2 border at the anterior limit of r4 by the five-somite stage can re-establish an isthmic organizer and partially rescue Gbx2 mutant phenotypes in Gbx2+/− containing the HG transgene. We show that the normal spatial relationship of Wnt1 and Fgf8 is restored at the new Otx2/Gbx2 border in Gbx2+/−; HG embryos, demonstrating that juxtaposition of Otx2 and Gbx2 after the five-somite stage is sufficient to re-establish the spatial expression of Wnt1 and Fgf8 in Gbx2+/− embryos. Despite co-expression of Fgf8 and Gbx2 in r4, the cerebellum fails to develop in Gbx2+/−; HG embryos. Thus, although Gbx2 is required for development of the cerebellum before the five-somite stage, other factors are required for mediating Fgf8 signaling during cerebellum development. In Gbx2+/−; HG embryos, the expression domain of Fgf8 is more restricted, whereas it is expanded in conditional Gbx2 mutants (Gbx2-CKO) in which Gbx2 is removed after E9.0 (Li et al., 2002). Interestingly, these two opposite alterations of Fgf8 expression are both associated with abnormal cell death in the posterior midbrain of Gbx2+/−; HG and Gbx2 CKO embryos, suggesting that cell survival in the posterior midbrain is positively or negatively regulated by Fgf8, depending on expression levels. Finally, we show that deletion of Gbx2 disturbs development of r4-6, and that expression of Gbx2 in r4 of Gbx2+/− embryos rescues development of r3, but not r1-2 or r4-6, uncovering a new role for Gbx2 in posterior hindbrain development.

Materials and methods

Generation of Hoxb1-Gbx2 transgenic mice

To generate the r4-Gbx2-Z transgene construct (Fig. 2A), a 0.8 kb SmaI/EcoRI DNA fragment bearing the minimal promoter of the mouse heat shock gene, hsp68 (Kohty et al., 1989) was cloned upstream of a full-length mouse Gbx2 cDNA (1.0 kb). A 330 bp EcoRI/HindIII DNA fragment containing the mouse Hoxb1 r4 enhancer (Popperl et al., 1995) was then placed upstream of the hsp68-Gbx2 insert. Finally, an IRES-lacZ with a polyadenylation signal sequence derived from SV40 (from plasmid pSP-NTR-nlacZ, a gift from Dr Maki Wakamiya) was placed immediately downstream of the Gbx2 cDNA.

Generation of a second Hoxb1-Gbx2 transgene was based on a 7.5 kb EcoRV mouse genomic DNA fragment containing the Hoxb1 r4 enhancer upstream to the endogenous promoter and most of the Hoxb1 locus (Popperl et al., 1995). A 187 bp HindIII/Eagl fragment flanking the translation start site was replaced with a truncated 90 bp HindIII/Eagl fragment generated by PCR to remove two In-frame ATG sites (pHoxb1-ΔATG). A 1.0 kb EcoRI/Asp718 Gbx2 cDNA was subsequently cloned into the Eagl site of pHoxb1-ΔATG. This transgene was designated r4-Gbx2 (Fig. 2A).

The r4-Gbx2-Z (7.0 kb) and r4-Gbx2 (8.4 kb) transgenes were released from the plasmid vectors by SmaI or EcoRV digest, respectively, and isolated by electrophoresis. These DNA fragments were further purified by dialysis against microinjection buffer and injected into mouse zygotes according to standard procedures (Nagy, 2003).

Histological analysis

Embryos were dissected in PBS and fixed in 4% paraformaldehyde in PBS at 4°C overnight. Embryos were processed for paraffin or frozen sectioning according to standard procedures (Nagy, 2003). For whole-mount in situ analysis, embryos were dehydrated and stored in methanol at ~20°C. Whole-mount RNA in situ hybridization was performed based on methods described previously (Wilkinson, 1992). Expression of lacZ in the transgenic embryos was analyzed by X-gal staining according to established protocols (Nagy, 2003). RNA in situ hybridization on paraffin or frozen sections was performed according to previously (Wassarman et al., 1997). The antisense RNA probes were as described previously: Fgf8 (Crossley and Martin, 1995), Gata2 (Pata et al., 1999), Gbx2 (Bouillet et al., 1995), Hoxa2 and Hoxb1 (Wilkinson et al., 1989b), kreisler (Mafb –
Development in the hindbrain

Gbx2 function in hindbrain development

Mouse Genome Informatics) (Cordes and Barsh, 1994), Krox20 (Egr2 – Mouse Genome Informatics) (Wilkinson et al., 1989a), Otx2 (Ang et al., 1994), Spry1 (Minowada et al., 1999), and Wnt1 (Parr et al., 1993).

Immunohistochemistry

Whole-mount embryo immunostaining with 2H3 antibody supernatant (Developmental Studies Hybridoma Bank, U. Iowa) was performed as described (Nagy, 2003).

BrdU cell proliferation assay and TUNEL assay

BrdU cell proliferation assay was performed as previously described (Mishina et al., 1995). Pregnant females were intraperitoneally injected with 100 µg BrdU per gram of body weight 1 hour before they were sacrificed. For the TUNEL assay, paraffin sections of embryos were dewaxed and apoptosis was detected with ApopTag (Serologicals, Norcross, GA) according to the manufacturer’s instructions.

Results

Ectopic expression of Gbx2 in r4 under the control of Hoxb1 enhancers

Based on morphological landmarks it was suggested that the expression domain of Otx2 ends immediately anterior to r4 in Gbx2 mutant embryos at E8.5 (Millet et al., 1999). To precisely define the posterior limit of Otx2 in Gbx2 mutants at early somite stages, we analyzed the expression of Otx2 and marker genes for r3 and r4 on adjacent sections. In wild-type embryos at the five-somite stage, Otx2 is expressed in the forebrain and midbrain, Hoxb1 in r4, and Krox20 in both r3 and r5 (Fig. 1A,D,G). The expression domains of Otx2 and Hoxb1 are clearly separated in wild-type embryos. By contrast, the expression domain of Otx2 was expanded posteriorly and slightly overlapped with Hoxb1 in Gbx2 mutants by the five-somite stage (Fig. 1B,E; see Fig. S1C,D in the supplementary material). Krox20 expression was only detected in r5, posterior to the expression domain of Hoxb1, as shown previously (Fig. 1H) (Wassarman et al., 1997). Therefore, the expression domains of Otx2 and Hoxb1 are partially overlapped, at the anterior border of r4 in Gbx2 mutant embryos by the five-somite stage.

To explore the significance of the Otx2/Gbx2 interface in regulating the isthmic organizer, we recreated an Otx2/Gbx2 border in Gbx2 mutants by expressing Gbx2 under the control of r4-specific enhancer elements of the mouse Hoxb1 gene. The enhancer used is responsible for maintaining expression of Hoxb1 in r4 through auto-regulation (Popperl et al., 1995). One Hoxb1-Gbx2 transgene, designated as r4-Gbx2, was generated by inserting the Gbx2 full-length cDNA in the 5′UTR of Hoxb1 in a 7.5 kb EcoRV mouse genomic fragment that includes all the Hoxb1 exons, endogenous promoter and the 330 bp r4 enhancer (Fig. 2A; see Materials and methods). In this transgene, the 5′UTR and downstream exon sequences of Hoxb1 will be transcribed together with the Gbx2 cDNA. A second Hoxb1-Gbx2 transgene, designated as r4-Gbx2-Z, was generated by inserting Gbx2-IRES-lacZ into a vector containing the 330 bp Hoxb1 r4 enhancer (Popperl et al., 1995) and the 0.8 kb hsp68 minimal promoter (Kothary et al., 1989) (Fig. 2A; see Materials and methods).

To analyze expression of the r4-Gbx2 transgene, we performed RNA in situ hybridization using a Gbx2 cDNA probe. Gbx2 was expressed in r1 and in two longitudinal stripes in the spinal cord of both wild-type and r4-Gbx2 transgenic embryos at E9.5 (Fig. 2B). As predicted, an additional Gbx2 expression domain was detected in r4 of the one r4-Gbx2 transgenic line examined (Fig. 2B). Similarly, ectopic Gbx2 expression was detected in r4 of the four r4-Gbx2-Z transgenic lines by RNA in situ hybridization or by analyzing the activity of β-galactosidase, which is translated from the bicistronic Gbx2-IRES-lacZ mRNA (Fig. 2C). Two r4-Gbx2-Z transgenic

Fig. 1. Partial overlapping of the expression domains of Otx2 and Hoxb1 in embryos lacking Gbx2 at the five-somite stage. (A-I) RNA in situ hybridization analysis of Otx2 (A-C), Hoxb1 (D-F) and Krox20 (G-I) on adjacent sagittal sections of embryos of indicated genotypes. Red arrowheads indicate posterior limit of Otx2 expression, and green arrowheads indicate the anterior limit of Hoxb1 expression. Arrows indicate r5. Inset in F shows that there are only a few cells in r4 (in bracket) expressing r4-Gbx2-Z after staining with X-gal.
lines (7 and 26) and the one r4-Gbx2 transgenic line (18) were selected for further analysis. The expression level of the transgene appeared from weak to high in the order of line 7, 18 and 26, and the expression level of Gbx2 in r4 was comparable with the endogenous expression in r1 in line 18 (Fig. 2B). In line 26 Gbx2/lacZ was also expressed in the mesenchyme near the isthmus (inset in Fig. 2C). Identical phenotypes were obtained with all three transgenic lines, which we refer to as HG transgenics (for Hoxb1-Gbx2) in the rest of the paper.

Transgenic mice with ectopic expression of Gbx2 in r4 develop normally

Hemizygous HG transgenics were viable and fertile, with no apparent phenotype. To examine whether ectopic expression of Gbx2 in r4 interferes with normal r4 development, we analyzed expression of Hoxb1 and Gata2. Gata2 is normally expressed in the ventral part of r4 and is a downstream target of Hoxb1 (Pata et al., 1999). The expression patterns of Hoxb1 and Gata2 were identical between wild-type and HG transgenic embryos at E9.5 (data not shown). We then examined whether ectopic expression of Gbx2 in r4 induced expression of genes that are normally expressed in r1. Fgf8 and Fgf17 were expressed normally in anterior r1 of HG transgenic, and no ectopic expression was detected in r4 (data not shown). Finally, to examine r4 development further in HG transgenics, we analyzed formation of the cranial nerves using neurofilament immunostaining. Cranial nerves VII (facial) and VIII (acoustic), which originate from r4, as well as other cranial nerves formed normally in HG transgenics (see Fig. S2 in the supplementary material). These data demonstrate that ectopic expression of Gbx2 in r4 is neither sufficient to transform r4 into r1 nor to interfere with r4 development.

Expression of Gbx2 in r4 of Gbx2 mutants rescues r3, but not r1, and leads to a loss of the posterior midbrain

To examine whether the HG transgenes can rescue any of the mutant phenotypes seen in Gbx2 mutants, we introduced the hemizygous HG transgene insertions onto a Gbx2-null mutant background (designated as Gbx2+/--; HG). In both wild-type and HG transgenic embryos at E10.5, the midbrain and hindbrain are clearly demarcated by an isthmic constriction, and dorsal r1 is composed of a bilaterally thickened neuroepithelium, which is the cerebellar primordium (Fig. 2D,E). In Gbx2 null mutants, the anterior hindbrain, including r1-3 is missing and the posterior limit of the expanded midbrain is juxtaposed with r4, which is located immediately anterior to the otic vesicles (Fig. 2F), as shown previously (Wassarman et al., 1997). By contrast, the distance between the posterior limit of the midbrain and the otic vesicles was significantly increased in Gbx2+/--; HG embryos at E10.5 (Fig. 2G), suggesting that the anterior hindbrain is partially rescued. However, the alar plate of r1 still appeared to be missing in Gbx2+/--; HG embryos. Similar to Gbx2-null mutants, Gbx2+/--; HG mutants died at birth. Morphological and histological analysis of embryos at E12.5 and E18.5 showed that the cerebellum did not form in Gbx2+/--; HG mutants, as in Gbx2+/--; embryos (Fig. 3). In contrast to an expanded midbrain in Gbx2+/--; embryos (Wassarman et al., 1997) (Fig. 3E,H), the posterior midbrain (inferior colliculus) was missing in Gbx2+/--; HG embryos (Fig. 3F,I). Therefore, although expressing Gbx2 in r4 of Gbx2 mutants partially rescues the anterior hindbrain, it does not rescue the cerebellum and leads to an additional loss of the posterior midbrain.

To identify the anterior hindbrain structure rescued by the HG transgenes in Gbx2 mutants, we analyzed expression of genes that demarcate the specific

---

**Fig. 2.** Expression of Gbx2 in rhombomere 4 using a Hoxb1 regulatory element partially rescues the anterior hindbrain of Gbx2−/− embryos. (A) Schematic representation of r4-Gbx2 and r4-Gbx2-Z transgenes. (B) Expression of Gbx2 analyzed by RNA in situ hybridization in r4-Gbx2 transgenic embryos and a wild-type control (inset) at E9.5. (C) Expression of r4-Gbx2-Z analyzed by X-gal staining. Inset shows a mid-sagittal section of an embryo. Gbx2 is ectopically expressed in r4 (black arrowhead) in r4-Gbx2 and r4-Gbx2-Z transgenic embryos. Gbx2/lacZ is also expressed in the head mesenchyme at the mid/hindbrain junction (black arrow) in r4-Gbx2-Z line #26. (D-G) Morphology of embryos of the indicated genotypes. The anterior hindbrain between the posterior limit of the mesencephalon and the otic vesicles (red arrowheads) are demarcated by a bracket. Asterisks indicate dorsal r1.
rhombomeres in the anterior hindbrain. In Gbx2\(^{-/-}\); HG embryos at the eight-somite stage, Krox20 was expressed in two distinct bands, as in wild-type embryos (Fig. 4A,C), whereas Krox20 is only expressed in r5 of Gbx2\(^{-/-}\) embryos (Fig. 4B), indicating r3 is rescued in Gbx2\(^{-/-}\); HG embryos. Interestingly, the transverse stripes of Krox20 were more restricted and the lateral-most expression of Krox20 in the neural plate was missing or greatly reduced in Gbx2\(^{-/-}\) or Gbx2\(^{-/-}\); HG embryos, suggesting Gbx2 is required for normal Krox20 expression. We next examined the expression of Hoxa2, which is expressed weakly in r2 and strongly in r3, r5 and in the neural crest cells migrating out from r4 of wild-type embryos at E9.5 (Fig. 4D). In Gbx2\(^{-/-}\) embryos the most anterior expression domain of Hoxa2 was previously found to be in the neural crest cells derived from presumptive r4 (Li and Joyner, 2001) (Fig. 4E), indicating the loss of r2 and r3 in these mutants. In contrast to Gbx2\(^{-/-}\) embryos, there was an additional transverse stripe of cells anterior to r4 strongly expressing Hoxa2 in Gbx2\(^{-/-}\); HG embryos (Fig. 4F), further demonstrating r3 is rescued in Gbx2\(^{-/-}\); HG embryos.

**R4-6 are abnormal in Gbx2-null mutants**

The abnormal expression of Krox20 in r5 of Gbx2\(^{-/-}\) and Gbx2\(^{-/-}\); HG mutants prompted us to analyze expression of another r5 marker, kreisler, which is specifically expressed in r5 and r6 at E8.5 (Cordes and Barsh, 1994) (Fig. 4G). We found that the transverse band of kreisler expression in r5-6 appeared narrower and the lateralmost expression in the neural plate was missing in Gbx2\(^{-/-}\) (Fig. 4H). The same alteration of kreisler expression was found in Gbx2\(^{-/-}\); HG embryos as in Gbx2\(^{-/-}\) embryos (Fig. 4I), demonstrating that r5 fails to rescue r5-6 development. As it has been shown that formation of r5-6 is regulated by r4 (Barrow et al., 2000; Maves et al., 2002; Walshe et al., 2002), we investigated whether r4 is formed normally in Gbx2\(^{-/-}\) embryos by analyzing expression of r4 marker genes. Follistatin is normally expressed along the paraxial mesoderm and in two transverse stripes in r1-2 and r4 at E8.5 (Albano et al., 1994; Feijen et al., 1994). In Gbx2\(^{-/-}\) embryos, both transverse bands of follistatin were found to be missing, whereas its expression in the paraxial mesoderm was normal (see Fig. S3B). Hoxb1 is normally expressed in a sharp transverse stripe throughout r4 at the six- to eight-somite stages (see Fig. S3C). In Gbx2\(^{-/-}\) embryos, the transverse band of Hoxb1 expression appeared expanded rostrally with a diffuse anterior border (see Fig. S3D). Furthermore, the lateralmost Hoxb1 expression in the neural plate was lost in Gbx2\(^{-/-}\) embryos, similar to the loss of the lateralmost expression of Krox20 and kreisler in r5 and r5-6, respectively. Therefore, this gene expression analysis demonstrates that deletion of Gbx2 disturbs development of r4-6.

**There is a duplication of r4 in Gbx2\(^{-/-}\); HG embryos**

As Gbx2 is required to inhibit Otx2 expression in r1-3 at the late headfold stage and thus allows development of r3 (Li and Joyner, 2001), we investigated whether the rescue of r3 in Gbx2\(^{-/-}\); HG embryos results from repression of Otx2 by the HG transgenes at the late headfold stage. Expression of the r4-Gbx2-Z transgene was therefore analyzed by X-gal staining between E7.75 and E8.5. At the headfold stage, r4-Gbx2-Z expression was found only in the posterior mesoderm within the primitive streak (data not shown). Expression of r4-Gbx2-Z in r4 was first detected at the five-somite stage (inset in Fig. 1F). Furthermore, at this stage the Otx2 expression was expanded posteriorly and partially overlapped with Hoxb1 in...
in Gbx2\(^{-/+}\); HG embryos at later stages by analyzing the expression of Otx2 and Hoxb1. In Gbx2\(^{-/+}\) embryos at E10.5, the expression domains of Otx2 and Hoxb1 were largely segregated with a few Otx2-positive cells in Hoxb1 expression domain (Fig. 5B,E). Interestingly, in Gbx2\(^{-/+}\); HG embryos Hoxb1 was expressed two separate stripes, a narrow band immediately posteriorly to the expression domain of Otx2 and its normal expression domain in r4 (Fig. 5C,F). The rostral transverse band was often discontinuous (Fig. 5F). The expression domains of the HG transgenes, analyzed by RNA in situ hybridization using a Gbx2 cDNA probe or X-gal staining, were identical to those of Hoxb1 in Gbx2\(^{-/+}\); HG embryos (inset in Fig. 5F; data not shown). Taken together, our marker gene analysis suggests that there is a duplication of r4 in Gbx2\(^{-/+}\); HG embryos, and that the tissue between the two Hoxb1 expression domains is probably r3-derived tissue rescued by the HG transgenes.

The HG transgenes restore the normal spatial relationship of Wnt1 and Fgf8 in Gbx2-null embryos

As a new Otx2/Gbx2 border region is established in Gbx2\(^{-/+}\); HG embryos at E10.5, we examined whether the new Otx2/Gbx2 border restored a functional isthmic organizer based on expression of the organizer genes Wnt1 and Fgf8. At E10.5, expression of Wnt1 and Fgf8 are normally restricted to narrow stripes on either sides of the Otx2/Gbx2 border (Fig. 5G,J), whereas the expression domains of Wnt1 and Fgf8 largely overlap in Gbx2\(^{-/+}\) embryos (Fig. 5H,K). In Gbx2\(^{-/+}\); HG embryos, Wnt1 and Fgf8 were largely expressed in complimentary domains, although Wnt1 expression expanded caudally in patches where Hoxb1/Gbx2 expression was missing (Fig. 5LL), suggesting a negative regulation of Wnt1 by Gbx2. This result demonstrates that the normal spatial relationship of Wnt1 and Fgf8 expression is restored specifically where a new Otx2/Gbx2 border is formed in Gbx2\(^{-/+}\); HG embryos.

Abnormal Fgf signaling in the mid/hindbrain junction region correlates with abnormal cell death in the posterior midbrain of Gbx2\(^{-/+}\); HG and Gbx2 conditional mutant embryos

An unexpected phenotype in Gbx2\(^{-/+}\); HG embryos was the loss of posterior midbrain tissue. To examine the mechanisms leading to this loss of posterior midbrain in Gbx2\(^{-/+}\); HG embryos, we first analyzed cell proliferation. Using a BrdU-immunohistochemistry assay, we compared cell proliferation in the posterior midbrain of wild-type, Gbx2\(^{-/+}\) and Gbx2\(^{-/+}\); HG embryos at E10.5 and E12.5. There was no obvious difference in cell proliferation among embryos of these

---

**Fig. 4.** R3 is rescued in Gbx2-null mutants by the HG transgenes. (A-C) Krox20 expression in embryos of the indicated genotypes at the eight-somite stages. Inset shows dorsal views of the embryo. The transverse bands of Krox20 expression in Gbx2\(^{-/+}\) (B) or Gbx2\(^{-/-}\); HG (C) embryos is narrower than those in the wild-type embryo (A). In addition, the lateral-most expression of Krox20 (arrowhead) is missing or greatly reduced in Gbx2\(^{-/-}\) and Gbx2\(^{-/+}\); HG embryos. (D-F) Hoxa2 expression at E9.5. Expression of Krox20 and Hoxa2 in r3 is restored in Gbx2\(^{-/+}\); HG embryos by the eight-somite stage and E9.5, respectively. (G-I) Kreisler expression in embryos at the six-somite stage of the indicated genotypes. Kreisler expression in r5-6 appears more restricted in Gbx2\(^{-/+}\) (H) and Gbx2\(^{-/-}\); HG (I) embryos, and the expression in the dorsal-most region (arrow) is missing.

Gbx2\(^{-/-}\); HG embryos by the five-somite stage, similar to that in Gbx2\(^{-/+}\) embryos, (Fig. 1G,F; see Fig. S1E-F in the supplementary material). These observations demonstrate that the rescue of r3 seen in Gbx2\(^{-/+}\); HG embryos by the eight-somite stage cannot be due to direct repression of Otx2 by Gbx2 in r3 at the five-somite stage.

To investigate the timing of rescue of r3 by ectopic expression of Gbx2 in r4, we examined the initiation of Krox20 expression in Gbx2\(^{-/+}\); HG embryos. Krox20 is normally initiated in r3 at the late headfold stage, and by the three-somite stage Krox20 is strongly expressed in r3 and weakly in r5 (Wilkinson et al., 1989a). In Gbx2\(^{-/+}\); HG embryos at the five-somite stage, although expression of Krox20 was clearly detected in r3, there were only a few Krox20-positive cells in presumptive r3, posterior to the expanded Otx2 expression domain (Fig. 1I). Therefore, development of r3 is delayed in Gbx2\(^{-/+}\); HG embryos.

We further examined development of the anterior hindbrain...
different genotypes (data not shown). We next examined apoptotic cell death using a TUNEL assay on sections of embryos at E10.5 and E12.5. TUNEL-positive cells were rarely detected in the mid/hindbrain region in wild-type and Gbx2-/- embryos (Fig. 6A,B), except in the dorsal midline region (data not shown). By contrast, in Gbx2-/-; HG embryos an increased number of TUNEL-positive cells was detected in the posterior region of the midbrain at E10.5 and E12.5 (Fig. 6C; data not shown). These data indicate that abnormal apoptosis probably contributes to the deletion of the posterior midbrain in Gbx2-/-; HG embryos. As Fgf8 has been shown to be essential for cell survival in the midbrain/r1 region (Chi et al., 2003), we investigated whether Fgf8 signaling is altered in Gbx2-/-; HG embryos. To visualize the spatial distribution of the Fgf8 expression domain in Gbx2-/-; HG embryos, we performed in situ RNA hybridization on whole-mount embryos. Compared with wild-type embryos, the expression domain of Fgf8 was broad and diffuse in E10.5 Gbx2-/- embryos (Fig. 6D,J). By contrast, Fgf8 was expressed in a sharp transverse band posterior to the prosencephalon in Gbx2-/-; HG embryos (Fig. 6K). However, the expression domain of Fgf8 was significantly narrower than that in wild-type or Gbx2-/- embryos. We next analyzed the expression of Spry1 as a readout of Fgf8 signaling, because we have shown that Spry1 can be directly induced by Fgf8 (Liu et al., 2003). Similar to the alteration of Fgf8, the expression domain of Spry1 was significantly more restricted than that in wild-type and Gbx2-/- embryos (Fig. 6E-G). These data thus demonstrate that Fgf8 signaling is reduced in Gbx2-/-; HG embryos.

We have previously generated a Gbx2 conditional mutant (Gbx2-CKO), in which Gbx2 is removed between E8.5 to E9.0 in the midbrain and r1 (Li et al. 2002). We observed that the midbrain in Gbx2-CKO mutants was often truncated at the dorsal midline, despite the posterior expansion of Otx2 into dorsal r1 (data not shown). In addition, the vermis, which is derived from anterior r1 (Sgaier et al., 2004). In Gbx2-CKO mice (Li et al., 2002). Therefore, we investigated whether abnormal cell death is involved in causing these phenotypes. Indeed we found a significant increase of TUNEL-positive cells r1 and the posterior midbrain on the parasagittal sections of E10.5 Gbx2-CKO embryos (Fig. 6D). Interestingly, in contrast to Gbx2-/-; HG embryos, there was an expansion and increase in the levels of Fgf8 and Spry1 expression in mid/hindbrain junction area of Gbx2 CKO embryos (Fig. 6L,H). Therefore, an increase of Fgf8 signaling is associated with increased cell death in the developing midbrain/r1 region of Gbx2-CKO embryos.

**Discussion**

**Gbx2 is not sufficient to induce a cerebellum**

Previous mutant studies have demonstrated that Gbx2 is essential for the specification of r1-3 by repressing Otx2 before E9.0 (Li and Joyner, 2001; Martinez-Barbera et al., 2001; Millet et al., 1999). In addition, gain-of-function experiments
showed that mis-expression of Gbx2 in the midbrain causes repression of Otx2 and anterior expansion of r1 (Katahira et al., 2000; Kikuta et al., 2003; Millet et al., 1999; Tour et al., 2002), and that this is dependent on the repressor function of Gbx2 (Tour et al., 2002). However, Gbx2 also has been shown to function as a transcriptional activator in certain cellular contexts (Kowenz-Leutz et al., 1997). Therefore, it remained unclear whether Gbx2 plays an additional positive role in directing r1-3 development. In this study, we expressed Gbx2 specifically in r4 of mouse embryos beginning at E8.5 using a Hoxb1 regulatory element. We show that r4 develops normally and no r1 markers are ectopically induced in r4 of the transgenic mice, demonstrating that Gbx2 is not sufficient to alter r4 development. Furthermore, by breeding the HG transgenics onto a Gbx2-null mutant background, we show that r4 is not transformed into a cerebellum, despite co-expression of Fgf8 and Gbx2 and formation of a new Otx2/Gbx2 border region after the five-somite stage. This demonstrates that Fgf8 and Gbx2 are not sufficient to induce a cerebellum in r4 after the five-somite stage. Taken together with previous studies, our results argue that Gbx2 mainly functions as a transcriptional repressor to regulate Otx2 expression and generate an Otx2-negative domain in r1-3. Transcription factors other than Gbx2, probably including Irx proteins (Itoh et al., 2002; Matsumoto et al., 2004), act downstream of Fgf8 signaling to direct formation of the cerebellum.

**Temporal requirements of Gbx2 in cerebellum development and formation of the isthmic organizer**

By generating a Gbx2 conditional mutant, we have previously shown that the lateral cerebellum forms after Gbx2 is removed in r1 at E9.0, demonstrating a functional requirement for Gbx2 in cerebellum formation only before E9.0 (Li et al., 2002). Our current study extends the conditional mutant analysis by showing that reintroduction of Gbx2 after E8.5 does not rescue cerebellum development. Thus, Gbx2 function is probably required earlier than E9.0, at least before the five-somite stage, based on our present study.

Despite development of a cerebellum, removal of Gbx2 after E9.0 disrupts the normal spatial relationship of the expression domains of Wnt1 and Fgf8 (Li et al., 2002). Gbx2 was thus found to have two requirements in maintaining Wnt1 and Fgf8 expression after E9.0: one in repressing Wnt1 expression and the other in delineating a narrow Fgf8 domain (Li and Joyner, 2001; Li et al., 2002). In the present study, we show that the normal spatial relationship of Wnt1 and Fgf8 is restored by expressing Gbx2 in r4 after E8.5 in Gbx2-/-; HG embryos, demonstrating that juxtaposition of Otx2 and Gbx2 after the five-somite stage is sufficient to establish the normal border of Wnt1 and Fgf8 expression.

**Fgf8 signaling positively and negatively regulates cell survival in the mid/hindbrain region**

Although expression of Wnt1 and Fgf8 at the mid/hindbrain junction has commonly been used as an indicator of an active isthmic organizer, the functional significance of the spatial relationship of the two genes and consequences of any small alterations in the levels of expression of the two genes have not been addressed. In Gbx2-CKO mutants, the expression domains of Wnt1 and Fgf8 overlap abnormally, and Fgf8 expression expands posteriorly and is elevated (Li et al., 2002) (Fig. 6). We have previously shown that cell proliferation is reduced within the Fgf8-expressing cells in both wild-type and Gbx2-CKO embryos (Li et al., 2002). In this study, we have extended our previous analysis by showing that apoptosis is significantly increased in dorsal regions of the posterior midbrain and anterior r1 in Gbx2-CKO embryos. We therefore suggest that the loss of the dorsal posterior midbrain in Gbx2-CKO mutants is due to abnormal cell death, whereas the loss of the medial cerebellum derived from anterior r1 results from...
a combination of reduction of cell proliferation and an increase in cell death.

In contrast to the overlapping and enhanced expression of Fgf8 and Wnt1 in Gbx2-CKO embryos, the spatial relationship of Wnt1 and Fgf8 is restored at the mid/hindbrain junction in Gbx2−/−; HG embryos but the two domains are abnormally restricted. Similar to Fgf8, the expression domains of Fgfl7 and Spry1 are more restricted to the posterior midbrain of Gbx2−/−; HG embryos than in wild-type embryos at E10.5 (Fig. 6; data not shown), suggesting that Fgf8 signaling in mid/hindbrain junction area is reduced in Gbx2−/−; HG embryos. Interestingly, there is a significant increase in the number of apoptotic cells in the posterior midbrain of Gbx2−/−; HG embryos than in wild-type embryos at E10.5 (Fig. 6; data not shown), suggesting that Fgf8 signaling regulates cell survival in the mid/hindbrain region at both high and low levels of expression.

**Gbx2 is required for normal development of r4-6**

In this study, we have uncovered a new phenotype in the posterior hindbrain of Gbx2−/− mutants. We show that the transverse stripes of follistatin expression in r1-2 and r4 are missing in Gbx2−/− embryos at E8.5. In addition, the expression domains of Krox20 in r5 and kreasler in r5-6 of Gbx2−/− embryos at E8.5 are more restricted than in wild-type embryos, and the lateral-most expression domains of each gene are mostly missing in Gbx2−/− mutants. These results demonstrate that in addition to the loss of r1-3 in Gbx2 mutants, development of r4-6 is disturbed. Interestingly, expression of Gata2 in ventral r4 and Hoxa2 in r5 appears normal in Gbx2−/− mutants (Fig. 4; data not shown). Furthermore, formation of motoneurons derived from r4-6 (Wassarman et al., 1997) or the projections of these neurons analyzed by neurofilament immunolabeling (see Fig. S2) appears normal in Gbx2−/− mutants. Therefore, the developmental consequence of the abnormal expression of a subset of genes that specifically mark r4-6 in Gbx2 mutants remains to be determined.

Gbx2 is initially expressed throughout the posterior embryo in both the mesoderm and ectoderm at E7.5 and becomes restricted to r1-3 by E8.5. Interestingly, Gbx2−/−; HG embryos share the same r5/6 phenotype as Gbx2−/− embryos, although the HG transgenes are expressed in the posterior mesoderm of the primitive streak at E7.5. Therefore, the disruption of r5-6 in Gbx2 mutants is probably due to a loss of Gbx2 in the ectoderm or mesoderm at the level of r5-6, as the HG transgenes are not expressed there. Similar to a transient requirement for Gbx2 in cerebellum development, the transient expression of Gbx2 in r4-6 between E7.5 to E8.5 could be essential for normal development of r4-6. Alternatively, Gbx2 may act primarily in formation of the isthmic organizer, which in turn regulates patterning of r4-6 (Irving and Mason, 2000). Finally, the anomalies in r5-6 could be secondary to abnormal formation of r4 in Gbx2 mutants, as r4 has been shown to function as an organizing center in zebrafish that regulates development of the adjacent rhombomeres (Maves et al., 2002; Walshe et al., 2002). In agreement with this, we observed abnormal expression of follistatin and Hoxb1 in r4 of Gbx2−/− and Gbx2−/−; HG mutants.

**Ecotropic expression of Gbx2 in r4 rescues development of r3 in Gbx2 null mutants**

One of the interesting findings in this study is that expression of Gbx2 in r4 restores expression of Krox20 and Hoxa2 in r3 of Gbx2 mutants, and leads to a duplication of the Hoxb1 expression domain anterior to the rescued r3. As both Krox20 and Hoxa2 are expressed in r3 as well as r5, we examined expression of kreasler, which is specifically expressed in r5-6.
Expression of inhibin subunits and follistatin during postimplantation mouse development: decidual expression of activin and expression of follistatin in primitive streak, somites and hindbrain.

Development 128, 9479-9491.

Li, J. Y. and Joyner, A. L. (2001). Otx2 and Gbx2 are required for refinement of the antero-posterior compartment of the ectoderm adjacent to the midbrain-hindbrain boundary.

Development 127, 177-186.

A role for ir01 and ir07 in the establishment of an anteroposterior compartment of the ectoderm close to the mid-hindbrain boundary.

Development 126, 707-714.

Expression of activin subunits, activin receptors and follistatin in postimplantation mouse embryos suggests specific developmental functions for different activins.

Development 120, 3621-3637.


Development 120, 433-450.


Development 105, 707-714.


Cell 91, 185-195.


Development 128, 4979-4991.


Neuron 36, 31-43.

Li, A., Li, J. Y., Bronleewe, C., Lao, Z., Niswander, L. A. and Joyner, A. L. (2003). FGFlb and FGFl8 have different midbrain regulatory properties from FGFl8b or activated FGF receptors.

Development 130, 6175-6185.


Science 274, 1109-1115.


Nature 360, 737-741.


Martinez, S., Crossley, P. H., Cobos, I., Rubenstein, J. L. and Martin, G. R. (1999). FGFl8 induces formation of an ectopic ishnic organizer and
ismocerebellar development via a repressive effect on Otx2 expression.
Development 126, 1189-1200.


