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

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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

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mutants (Wassarman et al., 1997), and *Otx2* expression appears to be extended to the anterior limit of r4 by E8.5 (Millet 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* for development of r1-3 by repressing *Otx2*, 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 reinstate 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 *Sma*I/*Nco*I DNA fragment bearing the minimal promoter of the mouse heat shock gene, *hsp68* (Kothary et al., 1989) was cloned upstream of a full-length mouse *Gbx2* cDNA (1.0 kb). A 330 bp *Eco*RI/*Hin*dIII DNA fragment containing the mouse *Hoxb1* r4 enhancer (Popperl et al., 1995) was then placed upstream of the *hsp68-Gbx2* insert. Finally, an *IRES-nlacZ* 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 *Eco*RV 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 *Hin*dIII/*Eag*I fragment flanking the translation start site was replace with a truncated 90 bp *Hin*dIII/*Eag*I fragment generated by PCR to remove two in-frame ATG sites (*pHoxb1-ΔATG*). A 1.0 kb *Eco*RV/*Asp*718 *Gbx2* cDNA was subsequently cloned into the *Eag*I 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 *Sma*I or *Eco*RV 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 methods described 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* –

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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 *Eco*RV 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

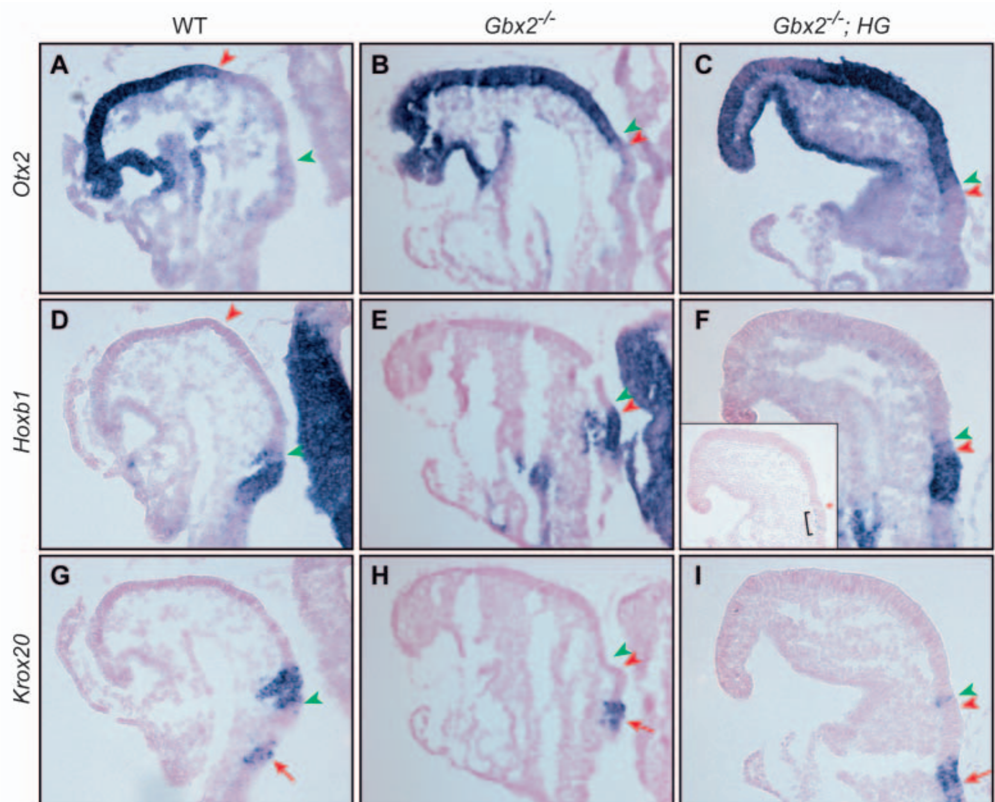

Fig. 1. Partial overlapping of the expression domains of *Otx2* and *Hoxb1* in embryos lacking *Gbx2* at the five-somite stage. (A-I) RNA in situ hybridization analysis of *Otx2* (A-C), *Hoxb1* (D-F) and *Krox20* (G-I) on adjacent sagittal sections of embryos of indicated genotypes. Red arrowheads indicate 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.

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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.

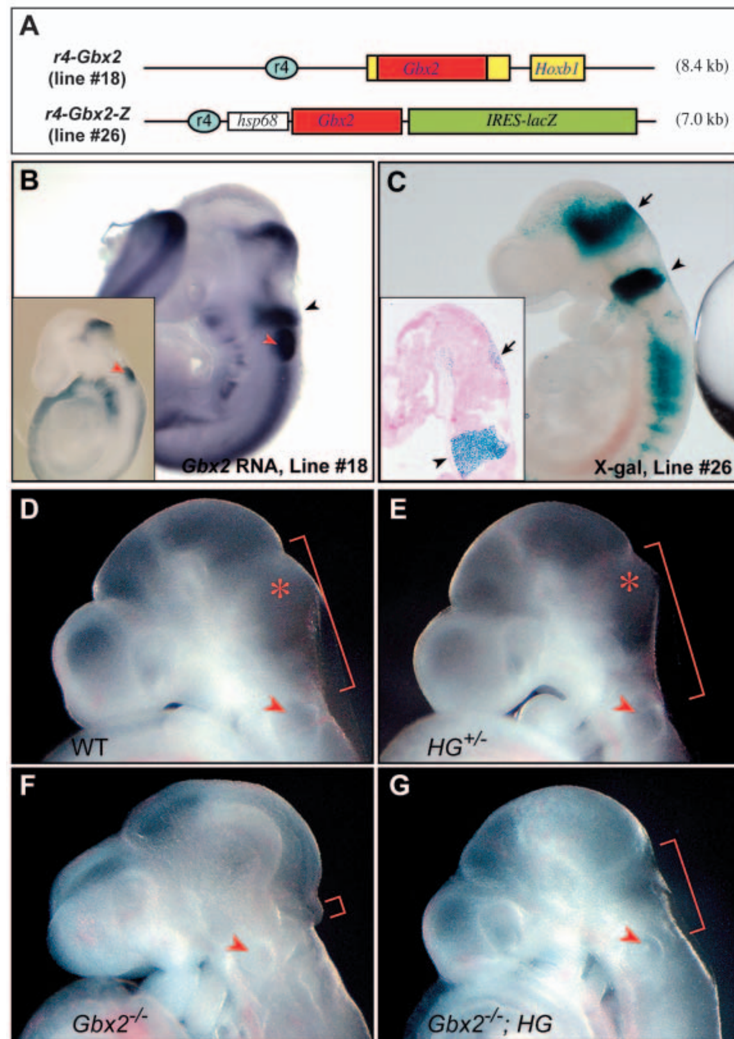

Fig. 2. Expression of *Gbx2* in rhombomere 4 using a *Hoxb1* regulatory element partially rescues the anterior hindbrain of *Gbx2*$^{-/-}$ embryos. (A) Schematic representation of *r4-Gbx2* and *r4-Gbx2-Z* transgenes. (B) Expression of *Gbx2* analyzed by RNA in situ hybridization in *r4-Gbx2* transgenic 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.

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

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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.

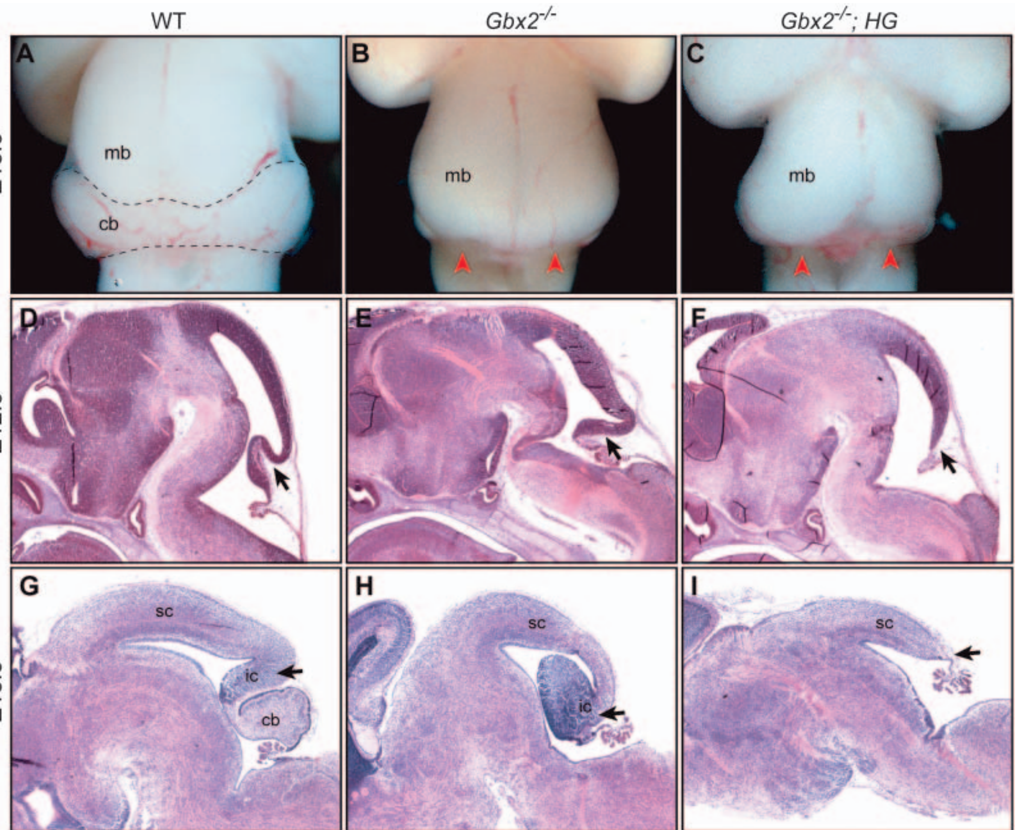

Fig. 3. The cerebellum and the posterior midbrain are missing in *Gbx2*$^{-/-}$*; HG* embryos. (A-C) Dorsal view of whole-mount brains in embryos of the indicated genotypes at E18.5. The cerebellum, which is demarcated by a broken line in the wild-type embryo, is absent in both *Gbx2*$^{-/-}$ and *Gbx2*$^{-/-}$*; HG* embryos (indicated by arrowheads). (D-I) Sagittal sections of embryos of the indicated genotypes at E12.5 (D-F) and E18.5 (G-I). The posterior midbrain (arrows) is missing in *Gbx2*$^{-/-}$*; HG* embryos. cb, cerebellum; mb, midbrain; sc, superior colliculus, ic, inferior colliculus.

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 expression of *Gbx2* in r4 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

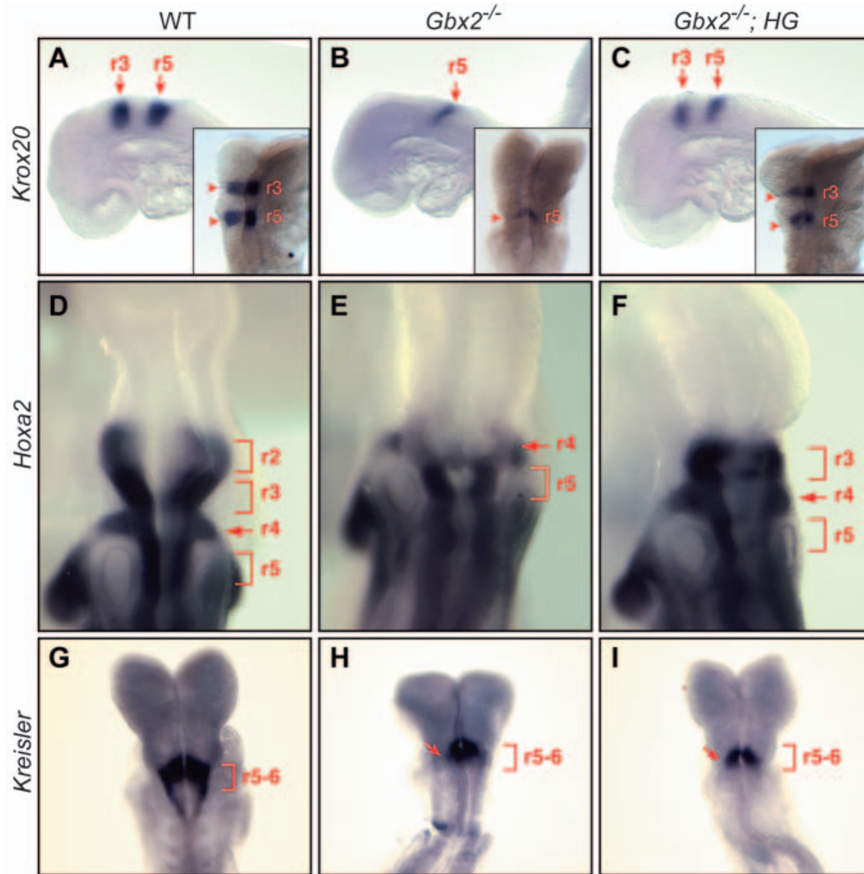

Fig. 4. R3 is rescued in *Gbx2*-null mutants by the *HG* transgenes. (A-C) *Krox20* expression in embryos of the indicated genotypes at the eight-somite stages. Inset shows dorsal views of the embryo. The transverse bands of *Krox20* expression in *Gbx2*$^{-/-}$ (B) or *Gbx2*$^{-/-}$*; HG* (C) embryos 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 r5, 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 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. 5I,L), 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

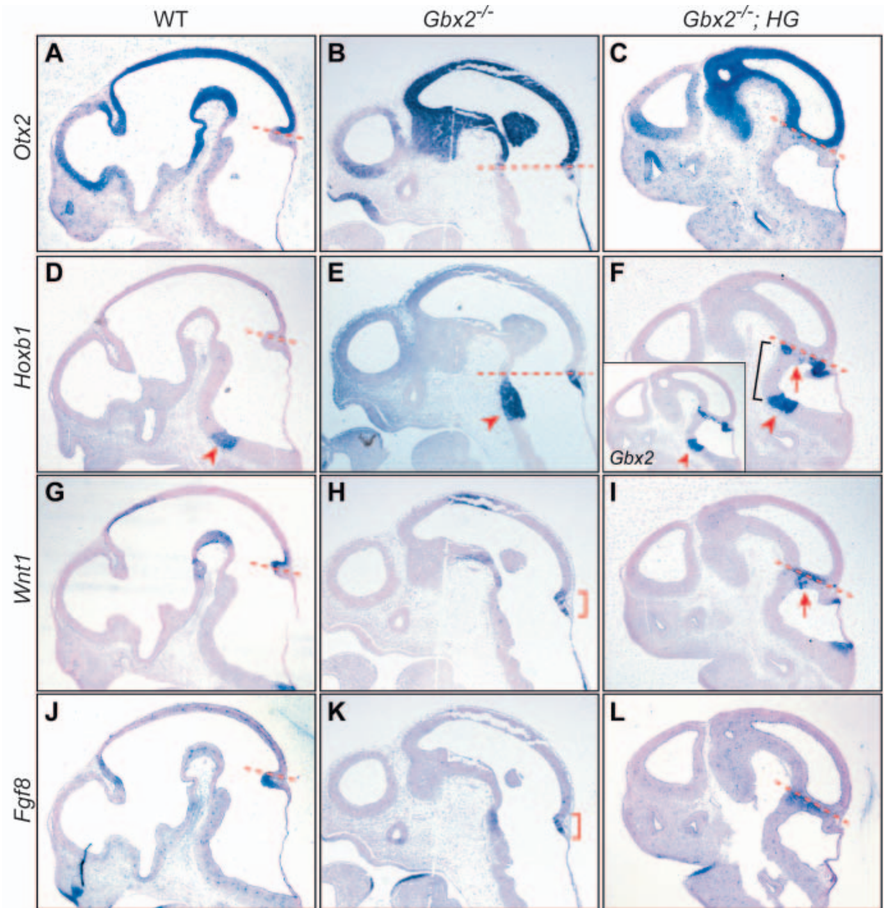

Fig. 5. A normal spatial relationship of *Wnt1* and *Fgf8* expression is restored in *Gbx2*-null mutants by the *HG* transgenes. (A-L) RNA in situ hybridization on sagittal sections of embryos of the indicated genotypes at E10.5. cDNA probes used for hybridization are listed to the left. Expression of *Otx2* and *Hoxb1* is juxtaposed in *Gbx2* mutant embryos (B,C,E,F), and ectopic *Otx2*-expressing cells (black arrowhead) are seen in *Hoxb1* expression domain of *Gbx2*$^{-/-}$ embryos (B). There is an additional transverse band of cells expressing *Hoxb1* in *Gbx2*$^{-/-}$*; HG* embryos (F). The region (indicated by a black bracket) between these two *Hoxb1* expression domains probably represents a rescued r3 tissue. Inset in F shows RNA in situ hybridization with a *Gbx2* probe, indicating that expression of the *HG* transgene (*Gbx2*) recapitulates the endogenous *Hoxb1* expression. *Wnt1* and *Fgf8* are expressed in complimentary domains at the *Otx2/Gbx2* border region in wild-type and *Gbx2*$^{-/-}$*; HG* embryos (G,I,J,L), whereas the expression domains (indicated by red bracket) of *Wnt1* and *Fgf8* largely overlap in *Gbx2* null embryos (H,K). Overlapping expression of *Wnt1* and *Fgf8* occurs specifically at a gap of *Hoxb1* (*Gbx2*) expression (marked by a arrow) in *Gbx2*$^{-/-}$*; HG* embryos. Broken lines demarcate the posterior limit of *Otx2* expression, read arrowhead indicates r4.

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. 6I,J). By contrast, *Fgf8* was expressed in a sharp transverse band posterior to the mesencephalon 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), is deleted 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 in 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

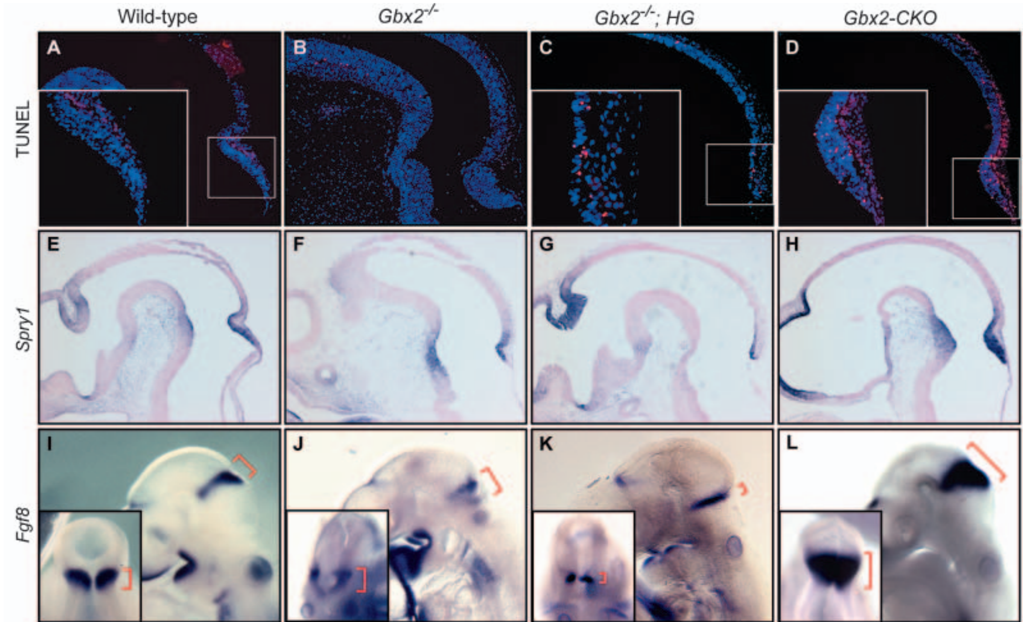

Fig. 6. Reduction or increase of *Fgf8* signaling in the mid/hindbrain junction leads to increased cell death in the posterior midbrain. (A-D) Analysis of apoptosis by a TUNEL assay on sagittal sections of E10.5 embryos of the indicated genotypes. There is a significant increase in the number of TUNEL-positive cells (red) in the posterior midbrain of *Gbx2*$^{-/-}$*; HG* (C) and r1 of *Gbx2*-CKO embryos (D). Insets in A, C and D show a higher magnification of the demarcated region. (E-L) Analysis of *Spry1* (E-H) and *Fgf8* (I-L) expression by RNA in situ hybridization in sections or whole-mount embryos of the indicated genotypes at E10.5. Insets in I-L show dorsal views of embryos. The expression domains of *Fgf8* and *Spry1* are diffuse in the mid/hindbrain region of *Gbx2*$^{-/-}$ embryos, and expanded in *Gbx2*-CKO embryos. By contrast, the expression domains of these genes are sharp but more restricted in *Gbx2*$^{-/-}$*; HG* embryos.

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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

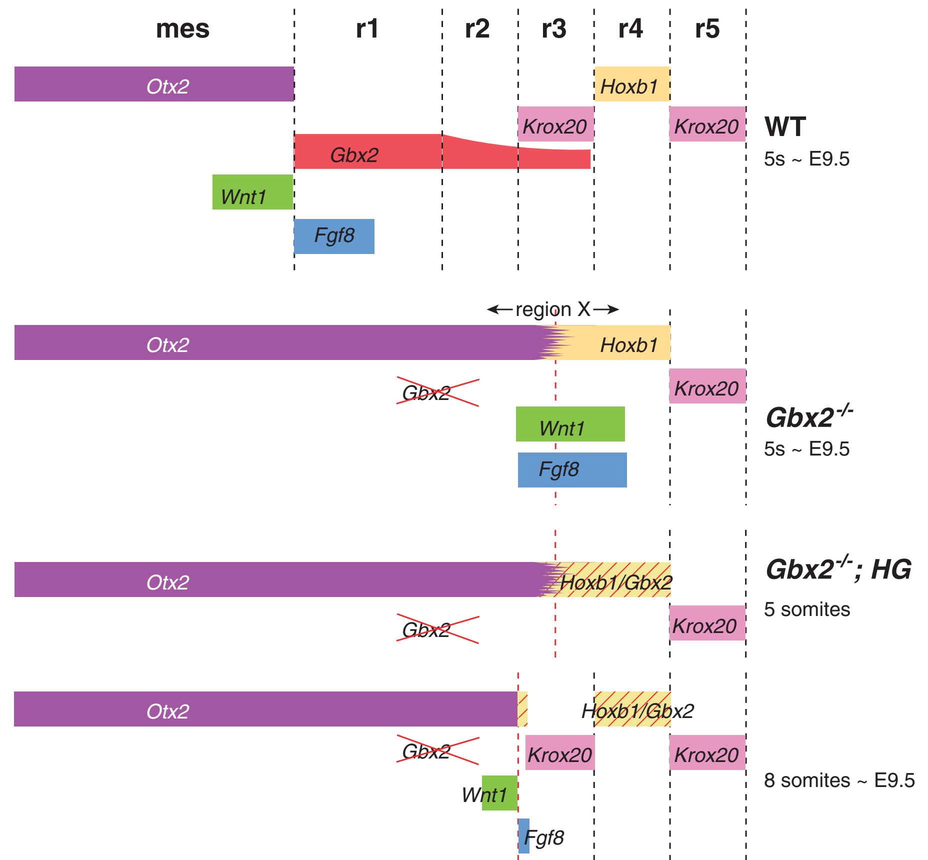

Fig. 7. Schematic summary of the gene expression. In *Gbx2*$^{-/-}$ embryos, there is an ill-defined area designated as region X, in which *Otx2*, *Hoxb1* are ectopically expressed. In contrast to wild type, expression domains of *Wnt1* and *Fgf8* overlap in region X. In *Gbx2*$^{-/-}$*; HG* embryos, the expression of *Gbx2* mimics *Hoxb1* expression at the five-somite stage and leads to repression of *Otx2* and a new *Otx2/Hoxb1* (*Gbx2*) border in region X at the eight-somite stage. The normal spatial relationship of *Wnt1* and *Fgf8* is established at the new *Otx2/Hoxb1* (*Gbx2*) border. *Krox20* is induced in r3 between two stripes of *Hoxb1* expression.

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 *Fgf17* 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, but no obvious change in cell proliferation (Fig. 6; data not shown). Our analysis of *Gbx2*-CKO and *Gbx2*$^{-/-}$*; HG* mutants therefore demonstrate that similar to a paradoxical control of cell survival by *Fgf8* in the developing forebrain (Storm et al., 2003), 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 kreisler 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.

Ectopic 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 kreisler, which is specifically expressed in r5-6

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at E8.5. We show that kreisler is only expressed in r5-6, but not in the rescued rhombomere in *Gbx2*$^{-/-}$*; HG* embryos. These results, thus indicate that r3 is rescued in *Gbx2*$^{-/-}$*; HG* embryos.

A previous study of *Gbx2* mutants showed that an ill-defined region is present at E10.5 between the midbrain and r4 of *Gbx2* mutant embryos designated as zone X (Wassarman et al., 1997). In zone X of *Gbx2*$^{-/-}$ embryos, *Otx2*, *Wnt1*, *Fgf8* and *Hoxb1* are abnormally co-expressed (Wassarman et al., 1997) (Figs 1, 5 and 7). We observed that expression of *Otx2* and *Hoxb1* overlaps in *Gbx2*$^{-/-}$ and *Gbx2*$^{-/-}$*; HG* embryos by the five-somite stage (Fig. 1 and see Fig. S1 in the supplementary material). We speculate that when the *Gbx2* cDNA is driven by the *Hoxb1* r4 enhancer in *Gbx2*$^{-/-}$*; HG* embryos, *Otx2* is repressed by Gbx2 in the zone X cells that ectopically express *Hoxb1*. A stable *Otx2/Hoxb1* (*Gbx2*) border is then established anterior to zone X in *Gbx2*$^{-/-}$*; HG* embryos at the five-somite stage, and subsequently *Krox20* is induced in zone X cells in between the new *Otx2/Hoxb1* (*Gbx2*) border and r4 (see summary in Fig. 7). We have previously shown that expression of *Krox20* and *Hoxa2* in r3 is restored in embryos lacking both *Gbx2* and *Otx2* after the eight-somite stage (Li and Joyner, 2001). However, it is not known whether *Krox20* expression in r3 is initiated normally at the 0- to 1-somite stage or, similar to that in *Gbx2*$^{-/-}$*; HG* embryos, is rescued later (at the 5- to 6-somite stage) in *Gbx2*$^{-/-}$*; Otx2*$^{-/-}$ embryos. Interestingly, we observed a gap between the expression domains of *Otx2* and *Hoxb1* transiently in *Gbx2* mutants at the 3- to 4-somite stage (see Fig. S1A,B in the supplementary material). Therefore, *Gbx2* may be essential for the initiation of *Krox20* expression in r3, whereas removal of *Otx2* in zone X in *Gbx2*-deficient embryos allows regeneration of r3 in *Gbx2*$^{-/-}$*; Otx2*$^{-/-}$ embryos or *Gbx2*$^{-/-}$*; HG* embryos after the five-somite stage.

The precise mechanism that leads to induction of *Krox20* in r3 of *Gbx2*$^{-/-}$*; HG* embryos at the five-somite stage remains to be elucidated. However, it is worth noting that following exposure of embryos to exogenous retinoid acid (RA), or mis-expression of *Hoxa1* in anterior rhombomeres, there is a transient expansion of *Hoxb1* but *Hoxb1* is maintained only in duplicated r4, and *Krox20* is subsequently induced in r3 between two *Hoxb1* expression domains (Alexandre et al., 1996; Marshall et al., 1992; Zhang et al., 1994). Therefore, an intrinsic mechanism apparently exists whereby *Hoxb1* is not maintained in r3 allowing expression of *Krox20*.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/8/1971/DC1

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