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Otx2 and *Gbx2* are required for refinement and not induction of mid-hindbrain gene expression

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SUMMARY

***Otx2* and *Gbx2* are among the earliest genes expressed in the neuroectoderm, dividing it into anterior and posterior domains with a common border that marks the mid-hindbrain junction. *Otx2* is required for development of the forebrain and midbrain, and *Gbx2* for the anterior hindbrain. Furthermore, opposing interactions between *Otx2* and *Gbx2* play an important role in positioning the mid-hindbrain boundary, where an organizer forms that regulates midbrain and cerebellum development. We show that the expression domains of *Otx2* and *Gbx2* are initially established independently of each other at the early headfold stage, and then their expression rapidly becomes interdependent by the late headfold stage. As we demonstrate that the repression of *Otx2* by retinoic acid is dependent on an induction of *Gbx2* in the anterior brain, molecules other than retinoic acid must regulate the initial expression of *Otx2* in vivo. In contrast to previous suggestions that an interaction between *Otx2*- and *Gbx2*-expressing cells may be essential for induction of mid-hindbrain organizer factors such as Fgf8, we find that *Fgf8* and other essential mid-hindbrain genes are induced in a correct temporal manner in mouse embryos deficient for both *Otx2* and *Gbx2*. However, expression of these genes is abnormally co-localized in a broad anterior region of the neuroectoderm. Finally, we find that by removing *Otx2* function, development of rhombomere 3 is rescued in *Gbx2*$^{-/-}$ embryos, showing that *Gbx2* plays a permissive, not instructive, role in rhombomere 3 development. Our results provide new insights into induction and maintenance of the mid-hindbrain genetic cascade by showing that a mid-hindbrain competence region is initially established independent of the division of the neuroectoderm into an anterior *Otx2*-positive domain and posterior *Gbx2*-positive domain. Furthermore, *Otx2* and *Gbx2* are required to suppress hindbrain and midbrain development, respectively, and thus allow establishment of the normal spatial domains of *Fgf8* and other genes.**

Key words: Compartment, *Fgf8*, Mid-hindbrain organizer, Retinoic acid, *Wnt1*, Mouse

INTRODUCTION

The molecular mechanisms that control development of the midbrain and cerebellum are an excellent paradigm of how stepwise inductive events can lead to patterning of the neuroectoderm along the anteroposterior (AP) axis. The midbrain develops from the mesencephalon (mes), an early morphologically distinct subdivision of the neural tube, and the cerebellum derives from the most anterior region of the hindbrain, the metencephalon (met). Studies of formation of these two distinct brain structures have shown that during embryogenesis, development of the mesencephalon and metencephalon is coordinately regulated. After the initial regionalization of the primitive neuroectoderm, patterning of the mes-met region is thought to be further refined by a local organizing center formed at the mes-met junction. Heterotopic transplantation studies using chick-quail chimeras have demonstrated that the mes-met boundary region can induce an ectopic midbrain with appropriate AP pattern in the posterior forebrain or anterior midbrain, and ectopic cerebellar tissue in the posterior hindbrain (Alvarado-Mallart, 1993; Le Douarin, 1993). Recent studies have demonstrated that *Fgf8*, which is expressed in the mes-met junction, is an important component of the organizer activity (Joyner et al., 2000). Fgf8-soaked beads placed in the caudal forebrain or anterior midbrain of chick embryos induce ectopic midbrain and cerebellar development (Martinez et al., 1999; Crossley et al., 1996; Shamim et al., 1999). Furthermore, partial loss-of-function mutations in *Fgf8* disrupt midbrain and cerebellum development in the mouse and fish (Meyers et al., 1998; Reifers et al., 1998; Brand et al., 1996).

Embryological manipulations in chick embryos have demonstrated that Fgf8 can be induced by a juxtaposition of posterior forebrain or midbrain, and rhombomere 1 (r1) tissues (Irving and Mason, 1999; Hidalgo-Sanchez et al., 1999b). These observations strongly support a model proposed by

Meinhardt that the formation of an organizing center involves initial specification of two populations of cells in adjacent territories and subsequent induction of cells at the common border to express signaling molecules (Meinhardt, 1983). According to Meinhardt's model, the mid-hindbrain organizer would be established via differential specification of the midbrain and hindbrain.

Previous studies have shown that development of the midbrain and hindbrain requires two homeobox genes, *Otx2* and *Gbx2*. *Otx2* and *Gbx2* are expressed by the headfold stage in the anterior and posterior neuroectoderm, respectively, and their common border of expression later demarcates the presumptive mid-hindbrain junction (Ang et al., 1994; Bouillet et al., 1995; Wassarman et al., 1997). Mouse embryos lacking *Otx2* have gastrulation defects and fail to form the neural structures anterior to r3 (Acampora et al., 1995; Ang et al., 1996; Matsuo et al., 1995), primarily owing to a requirement for *Otx2* in the anterior visceral endoderm (Rhinn et al., 1998; Acampora et al., 1998; Kimura et al., 2000; Perea-Gomez et al., 2001). Embryos that lack *Otx2* function specifically in the epiblast and its derivatives, however, lack only a forebrain and midbrain (Acampora et al., 1995; Rhinn et al., 1998). In a complementary manner, *Gbx2* mutant embryos fail to develop anterior hindbrain structures including r1-3, and *Otx2* expression is expanded caudally at the four- to six-somite stages, showing that the anterior hindbrain is transformed into a midbrain fate (Millet et al., 1999; Wassarman et al., 1997).

Gain-of-function studies have demonstrated that mutual antagonism between *Otx2* and *Gbx2* determines the position of mid-hindbrain border. Misexpression of *Gbx2* leads to repression of *Otx2* in the posterior midbrain (Millet et al., 1999; Katahira et al., 2000), and similarly misexpression of *Otx2* results in repression of *Gbx2* in the metencephalon (Broccoli et al., 1999; Katahira et al., 2000). In both cases, the expression domain of *Fgf8* is shifted and situated at the new *Otx2*-*Gbx2* border. In agreement with Meinhardt's model, *Otx2* and *Gbx2* could therefore confer differential specification to the mesencephalic and metencephalic cells and subsequent interactions between these two populations of cells could lead to induction of *Fgf8* at their common border. Interestingly, in the absence of either *Otx2* or *Gbx2* alone, *Fgf8* is still expressed (Wassarman et al., 1997; Acampora et al., 1998; Rhinn et al., 1998). It could be, however that juxtaposition of *Otx2*- or *Gbx2*-expressing cells with *Otx2* or *Gbx2* non-expressing cells is sufficient to induce *Fgf8*.

As the domains of *Otx2* and *Gbx2* expression position the mid-hindbrain organizer, it is important to determine how their expression domains are established. *Otx2* is initially expressed throughout the epiblast of mouse embryos before gastrulation and its expression becomes progressively restricted to the anterior third of the embryo by the headfold stage (Ang et al., 1994). Expression of *Gbx2* is first detected at the mid-streak stage in the primitive streak (J. H. L. and A. L. J., unpublished), and as gastrulation proceeds expression extends laterally and anteriorly such that its anterior limit directly abuts the posterior domain of *Otx2* (Wassarman et al., 1997; Hidalgo-Sanchez et al., 1999a; Garda et al., 2001). These dynamic and complimentary expression patterns of *Otx2* and *Gbx2*, and the presence of an apparent antagonism between them suggest that an interaction between these two genes could directly regulate their expression domains in vivo. Intriguingly, in mouse embryos that lack *Otx2* in the epiblast, *Gbx2* expression appears normal at E7.75, based on section in situ analysis, but rostrally expanded at E8.5 (Acampora et al., 1998), while in *Gbx2* homozygous mutants, caudal expansion of *Otx2* was detected at the four- to six-somite stage but earlier stages were not analyzed (Millet et al., 1999). There are suggestions that *Fgf8*, which starts to be expressed at the three-somite stage, might actually mediate the apparent opposing interaction between *Otx2* and *Gbx2* (Acampora et al., 1997; Liu and Joyner, 2001; Wassarman et al., 1997). Therefore, further studies of the timing of gene alterations in *Otx2* or *Gbx2* mutant embryos at early stages could provide new insights into the molecular mechanism that underlies the interaction between *Otx2* and *Gbx2*, and the role of this interaction in regulating the expression domains of *Otx2* and *Gbx2*.

It also has been suggested based on a germ-layer-recombination assay in mouse that expression of *Otx2* during gastrulation is regulated by positive and negative signals from anterior and posterior mesoderm, respectively (Ang et al., 1994). Retinoic acid (RA), a posteriorizing factor, can repress *Otx2* expression in mouse embryos treated in utero at E7.5 (Ang et al., 1994), whereas *Gbx2* can be induced by RA in cultured P19 embryonal carcinoma cells and *Xenopus* embryos (Bouillet et al., 1995; von Bubnoff et al., 1996). However, it remains to be determined whether RA normally plays a crucial role in regulating expression of *Otx2* and *Gbx2* in vivo.

In order to determine whether an interaction between *Otx2* and *Gbx2* is required for determining their initial expression domains, we performed a detailed analysis of *Gbx2* and *Otx2* expression in embryos that lacked *Otx2* or *Gbx2*. We show that the expression domains of *Otx2* and *Gbx2* are initially established independently of each other, but that by the late headfold stage (LHF) antagonistic interactions between these two genes play a crucial role in maintaining their respective borders of expression at the mes-met junction. To investigate factors that could regulate the early expression pattern of *Otx2* and *Gbx2*, we analyzed expression of these two genes in mouse embryos treated in utero with RA. Expression of *Gbx2* is induced anteriorly within 4 hours of RA treatment, and *Otx2* is repressed in the midbrain and all but the anterior most forebrain by 24 hours. Significantly, in the absence of *Gbx2*, *Otx2* is not repressed by RA. Furthermore, to study the collective roles of *Otx2* and *Gbx2* in initiation of *Fgf8* and genes that are normally expressed in the mes-met region (mes-met genes), we generated *Otx2* and *Gbx2* double homozygous mutant embryos. We show that *Otx2* and *Gbx2* are not required for initiation or maintenance of these genes. *Otx2* and *Gbx2* are essential, however, for negatively regulating *Fgf8* and *Wnt1*, respectively, and thus subdividing the presumptive mes-met region into two different domains. Finally, given the apparent antagonistic interaction between *Otx2* and *Gbx2*, some of the phenotypes seen in *Otx2* or *Gbx2* single mutants could result primarily from mis-expression of *Gbx2* or *Otx2*, respectively, rather than a positive requirement for *Otx2* or *Gbx2*. Indeed, we show that *Gbx2* plays only a permissive role in r3 development, whereas *Otx2* is intrinsically required for forebrain development.

MATERIALS AND METHODS

Generation and genotyping of wild type and mutant mice

Noon of the day on which the vaginal plug was detected was considered as E0.5 in timing of embryos. Staging of embryos before somite formation was based on morphological landmarks (Downs and Davies, 1993). *Gbx2* and *Otx2* mutant mice were maintained on an outbred background. Genotypes of offspring were determined by PCR analysis as described (Acampora et al., 1998; Wassarman et al., 1997).

Retinoic acid treatment

Retinoic acid was administrated to pregnant females as described previously (Conlon and Rossant, 1995). All-*trans* retinoic acid (Sigma) was dissolved in DMSO (100 mg/ml) and further diluted to 10 mg/ml with corn oil before use. The mixture was administrated by oral gavage to a final dose of 20 μg/g of body weight of the pregnant females. The administration was performed between 10 am and noon on E7.5, and embryos were dissected and fixed 4, 8 or 24 hours after RA administration.

In situ hybridization

Whole-mount RNA in situ hybridization was performed essentially as previously described (Wilkinson, 1992). Section RNA in situ hybridization was performed as described (Wassarman et al., 1997). The antisense riboprobes for the following genes were used: *Bf1* (*Foxg1* – Mouse Genome Informatics) (Tao and Lai, 1992), *Otx2* (Ang et al., 1994), human *OTX1* (Acampora et al., 1998), *Hesx1* (Thomas and Beddington, 1996), *Gbx2* (Bouillet et al., 1995), *Fgf8* (Crossley and Martin, 1995), *Wnt1* (Parr et al., 1993), *En1* and *En2* (Millen et al., 1995), *Six3* (Oliver et al., 1995), *Krox20* (*Egr2* – Mouse Genome Informatics) (Wilkinson et al., 1989a), *Pax2* (Dressler and Douglass, 1992), *Hoxa2* and *Hoxb1* (Wilkinson et al., 1989b).

RESULTS

An interaction between *Gbx2* and *Otx2* is required to maintain their anterior and posterior expression limits, respectively, as early as the late headfold stage

To determine the requirement for an interaction between *Otx2* and *Gbx2* in regulating their expression domains, we performed detailed expression analysis of *Otx2* and *Gbx2* mutants using morphological landmarks to stage embryos from E7.0 to E8.5 (Downs and Davies, 1993). In order to study the requirement for *Otx2* in development of the anterior neuroectoderm, we used a mutant allele, $Otx2^{hOtx1}$, in which the human OTX1 protein is expressed in place of mouse Otx2 only in the anterior visceral endoderm, and rescues the gastrulation defects of *Otx2* null mutants (Acampora et al., 1998). The OTX1 protein is not produced from this allele in the epiblast and its derivatives, although *OTX1* mRNA transcripts are expressed from the *Otx2* locus (Acampora et al., 1998).

First, we double labeled for transcripts of *Gbx2* and *Hesx1*, a homeobox gene that is normally expressed in the prospective forebrain at the headfold stage (Thomas and Beddington, 1996). At the early headfold (EHF) stage, *Hesx1* was not detected in $Otx2^{hOtx1/hOtx1}$ embryos (n=3), whereas *Gbx2* was readily detected (Fig. 1A). The distance between the anterior limit of *Gbx2* expression and the position of the node in the midline of $Otx2^{hOtx1/hOtx1}$ embryos was comparable with that in wild-type controls. By contrast, by the LHF stage, *Gbx2* expression in $Otx2^{hOtx1/hOtx1}$ embryos was significantly expanded rostrally, based on an increased distance between the

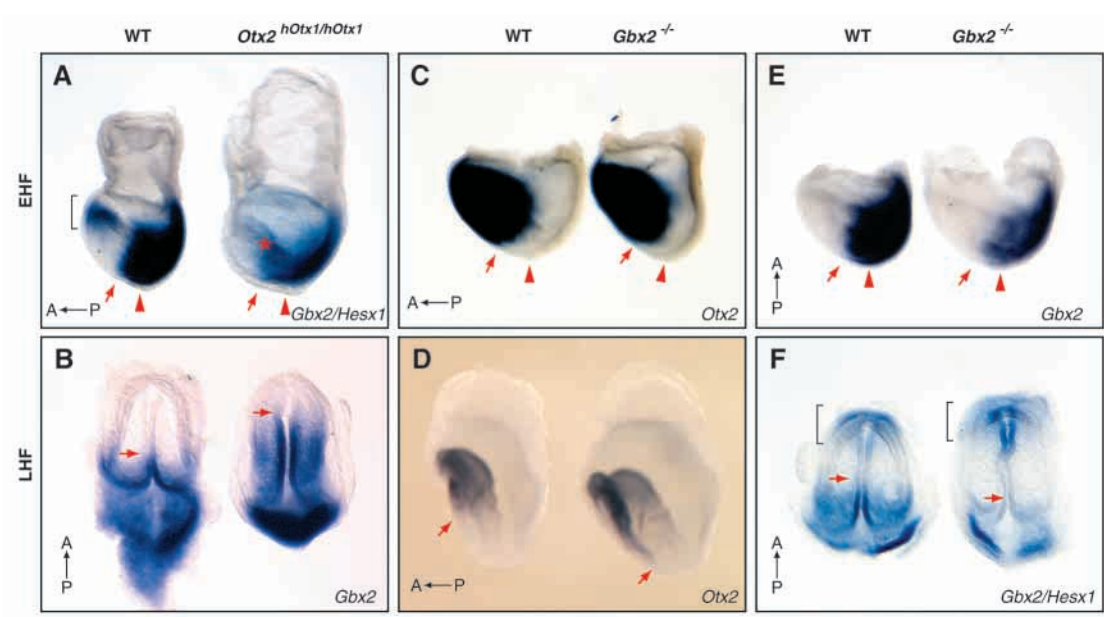

Fig. 1. An interaction between *Gbx2* and *Otx2* defines the limits of their respective expression domains at the start of somitogenesis. (A) Expression of *Gbx2* and *Hesx1* in wild-type and $Otx2^{hOtx1/hOtx1}$ embryos at the EHF stage. The anterior limit of *Gbx2* expression in the midline (arrow), relative to the position of the node (arrowhead) in $Otx2^{hOtx1/hOtx1}$ and wild-type embryos is comparable, although the lateral expression of *Gbx2* (asterisk) appears slightly expanded anteriorly in the mutant. (B) *Gbx2* expression is significantly expanded anteriorly in $Otx2^{hOtx1/hOtx1}$ embryos at the LHF stage, compared with wild-type controls. (C,D) Expression of *Otx2* in wild-type and $Gbx2^{-/-}$ embryos at the EHF (C) and LHF (D) stage. Relative to the position of the node (arrowhead), the posterior limit of *Otx2* (arrow) is not altered in $Gbx2^{-/-}$ embryos at the EHF stage, but shifted caudally by the LFH stage, particularly in the midline. (E) Expression of *Gbx2* is normal in $Gbx2^{-/-}$ embryos at the EHF stage. (F) *Hesx1* expression (brackets) is not changed in $Gbx2^{-/-}$ embryos, whereas *Gbx2* expression is reduced and its anterior limit (arrow) is shifted caudally compared with wild-type controls at the LHF stage. The apparently stronger staining of *Hesx1* in the $Gbx2^{-/-}$ embryo is due to a prolonged color reaction in the mutant compared with the wild type in order to visualize *Gbx2* staining. (B,F) Dorsal views of flat-mount embryos.

anterior limit of the *Gbx2* domain and the position of the node, as well as a reduced *Gbx2*-negative region in the anterior ectoderm (Fig. 1B). At the four-somite stage, *Gbx2* expression was expanded to the anterior tip of mutant embryos. By the six-somite stage, its expression became restricted to the anterior tip (data not shown) (Acampora et al., 1998). Thus, *Otx2* function is not required to determine the initial anterior expression limit of *Gbx2* but is required rapidly to maintain it. Furthermore, consistent with a previous study of chimeras composed of *Otx2* mutant and wild-type cells (Rhinn et al., 1998), *Otx2* is required for *Hesx1* expression in the anterior neuroectoderm at a time when it is not required to regulate *Gbx2*.

Our finding that *Gbx2* is expanded rostrally in $Otx2^{hOtx1/hOtx1}$ embryos by the LHF stage prompted us to examine whether *Gbx2* is also required to define the posterior limit of *Otx2* expression at the equivalent stage. *Otx2* is normally expressed at the EHF stage in the anterior third of embryos with a diffuse posterior limit. A similar pattern of expression was seen in the EHF stage $Gbx2^{-/-}$ embryos (Fig. 1C). However, at the LHF stage, expression of *Otx2* was abnormally expanded caudally, primarily in the midline of $Gbx2^{-/-}$ embryos (Fig. 1D).

To determine whether the caudal expansion of *Otx2* in $Gbx2^{-/-}$ embryos at the LHF stage is associated with a loss of r1-3 specification, we first examined expression of *Krox20*, which is initially expressed in r3 from the LHF to three-somite stages (Wilkinson et al., 1989a). In $Gbx2^{-/-}$ embryos, *Krox20* expression was not detected between the LHF and three-somite stages (n=3), whereas its expression was readily detected in r3 of wild-type controls (n=3) at the same stages (data not shown). *Krox20* expression also was not detected in r3 in $Gbx2^{-/-}$ embryos at the seven-somite stage (see Fig. 7B) (Wassarman et al., 1997). These observations show that *Gbx2* is required for initiation of *Krox20* expression in r3.

To further examine whether caudal expansion of *Otx2* results in a general respecification of the presumptive r1-3 region, we analyzed *Gbx2* RNA expression in $Gbx2^{-/-}$ embryos at the LHF stage, using a probe corresponding to 5′ coding sequences not deleted in the mutant allele (Wassarman et al., 1997). In $Gbx2^{-/-}$ embryos, expression of *Gbx2* was normal at the EHF stage (Fig. 1E). By contrast, at the late LHF stage in $Gbx2^{-/-}$ embryos, expression of *Gbx2* was significantly reduced and its anterior expression limit was shifted posteriorly (Fig. 1F). Interestingly, expression of *Gbx2* was greatly reduced in $Gbx2^{-/-}$ embryos at E8.5 (n=2), even in posterior regions of the embryo where development seems unaffected by disruption of *Gbx2*, suggesting *Gbx2* becomes autoregulated.

Taken together, these data demonstrate that establishment of the initial posterior or anterior limit of *Otx2* or *Gbx2* expression at the EHF stage is not dependent on *Gbx2* or *Otx2*, respectively, but by the LHF stage, an interaction between *Otx2* and *Gbx2* plays a crucial role in defining their respective expression limits. Disruption of either *Otx2* or *Gbx2* results in rapid expansion of the *Gbx2* or *Otx2* expression domains, respectively, which may then lead directly to respecification of the midbrain or r1-3.

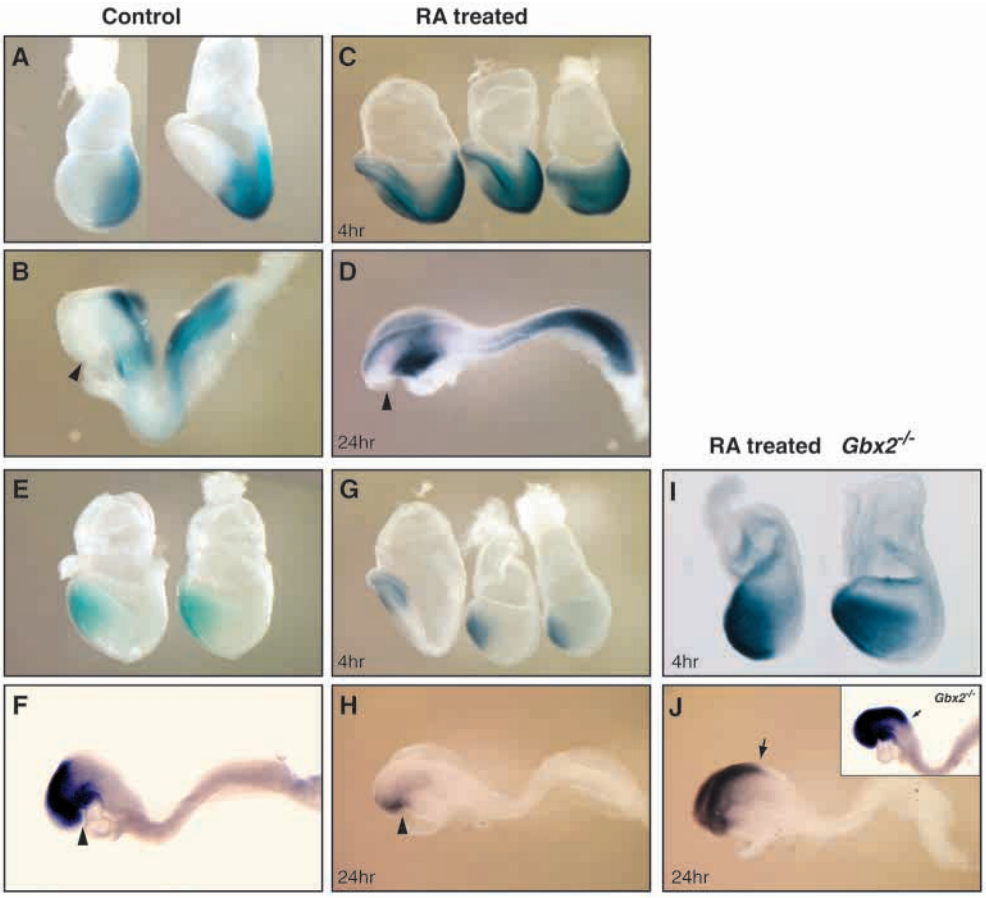

Fig. 2. *Gbx2* is required for repression of *Otx2* by exogenous RA. (A-D) Expression of *Gbx2* in wild-type control (A,B) and RA-treated embryos (C,D). (E-J) Expression of *Otx2* in a wild-type control (E,F) and RA-treated wild-type embryo (G,H) and RA-treated $Gbx2^{-/-}$ embryo (I,J). (C,G,I) Embryos 4 hours after RA treatment. (D,H,J) Embryos 24 hours after RA treatment. Note that by 4 hours, *Gbx2* is already induced anteriorly by RA (C), whereas *Otx2* is only significantly repressed by 24 hours and restricted to the most anterior tip (arrowhead) of the embryo (G,H). The repression of *Otx2* by RA is inhibited in $Gbx2^{-/-}$ embryos (I) and 24 hours after RA treatment *Otx2* is expanded posteriorly to the presumptive r3/4 border (arrow) (J), similar to that in untreated $Gbx2^{-/-}$ embryos at E8.5 (inset in J). Anterior is towards the left.

Gbx2 is required for repression of *Otx2* by exogenous RA

As the expression domains of *Otx2* and *Gbx2* are initially established independently of each other, it was of interest to explore what molecules might regulate their initial expression patterns. RA has been implicated as a posteriorizing factor during embryonic AP patterning, and has opposite effects on *Otx2* and *Gbx2* expression (Ang et al., 1994; Bouillet et al., 1995; von Bubnoff et al., 1996). Therefore, we studied the temporal and spatial responses of *Otx2* and *Gbx2* in mouse embryos 4, 8 and 24 hours after exposure to a teratogenic dose of RA.

Within 4 hours of RA treatment of mouse embryos at E7.5, expression of *Gbx2* was rapidly and dramatically induced and expanded anteriorly (Fig. 2A,C). As shown previously (Ang et al., 1994), the expression domain of *Otx2* was only slightly reduced in the anterior region of embryos 4 hours after RA treatment (Fig. 2E,G) and its expression domain was further reduced by 8 hours (data not shown). Twenty-four hours after RA treatment, *Gbx2* expression was found to be expanded and in a broad anterior region with a small *Gbx2*-negative domain at the anterior tip of the embryos (Fig. 2B,D). The *Gbx2*-negative region seemed to correspond to a greatly restricted *Otx2* expression domain (Fig. 2F,H).

The rapid induction of *Gbx2* by RA and an apparent antagonistic interaction between *Otx2* and *Gbx2* led us to investigate whether *Gbx2* is required for the RA-mediated repression of *Otx2*. Interestingly, expression of *Otx2* in $Gbx2^{-/-}$ embryos was not repressed 4 hours (Fig. 2I) or 24 hours (Fig. 2J) after exogenous RA treatment. Instead, in $Gbx2^{-/-}$ embryos with or without RA treatment, *Otx2* was expanded to the presumptive r3/4 border. These results demonstrate that *Gbx2* is required to mediate repression of *Otx2* by RA. The studies also indicate that the initial restriction of *Otx2* expression to the anterior region of the embryo does not depend on RA signaling, as the expression domain of *Otx2* is normal at the EHF stage in $Gbx2^{-/-}$ embryos.

Mes-met genes are induced in a normal temporal order, but in a broad anterior region in $Gbx2^{-/-}$; $Otx2^{hOtx1/hOtx1}$ embryos

Based on Meinhardt's model, the division of the neural ectoderm into anterior *Otx2*-positive and posterior *Gbx2*-positive domains could be imperative for the induction of *Fgf8* and other mes-met genes. To test this hypothesis, we generated $Gbx2^{-/-}$; $Otx2^{hOtx1/hOtx1}$ embryos and investigated whether the mes-met genes are induced in these double mutant embryos at early somite stages.

At the four-somite stage, *Fgf8* was detected in the metencephalon (Fig. 3A). In $Otx2^{hOtx1/hOtx1}$ embryos at the five-somite stage, diffuse *Fgf8* expression was detected in a broad domain of the anterior neuroectoderm and slightly stronger expression was seen at the anterior tip of the embryos (Fig. 3B). By the seven-somite stage, *Fgf8* expression became restricted to the anterior tip of the mutant embryos (inset in Fig. 3B) (Acampora et al., 1998). In $Gbx2^{-/-}$ mutants the expression of *Fgf8* in the presumptive metencephalon was greatly reduced and shifted caudally (Fig. 3C) and by the seven-somite stage *Fgf8* was diffuse and expanded to r4 and fused with expression of *Hoxb1*, a gene that normally marks r4 (inset in Fig. 3C). Interestingly, at both the four- and six-somite stages in $Gbx2^{-/-}$; $Otx2^{hOtx1/hOtx1}$ embryos, *Fgf8* was strong and throughout a large anterior domain (Fig. 3D and inset). Therefore, *Otx2* and *Gbx2* are not required for *Fgf8* to be induced.

We next examined another gene expressed at the mes-met junction. *Wnt1* is normally expressed across the entire mesencephalon at the four-somite stage and then in a narrow band anterior to the *Fgf8* expression domain (Fig. 3E). In $Otx2^{hOtx1/hOtx1}$ mutants at the five-somite stage, *Wnt1* was absent from the mesencephalon (n=2) (Fig. 3F). By the eight-somite stage *Wnt1* was found in the lateral edges of the neural fold along the entire AP axis (n=2) (inset in Fig. 3F) (Acampora et al., 1998). In $Gbx2^{-/-}$ embryos, *Wnt1* expression was expanded caudally at the five-somite stage (Fig. 3G) (Millet et al., 1999). In contrast to either single mutant, in $Gbx2^{-/-}$; $Otx2^{hOtx1/hOtx1}$ embryos at the four-somite stage, *Wnt1* expression was patchy and expanded to the anterior tip of the neuroectoderm overlapping with *Fgf8* (Fig. 3H).

Pax2 is the earliest known gene expressed in the mes-met region. *Pax2* transcripts are first detected in the anterior ectoderm of mouse embryos at the late streak stage (Rowitch and McMahon, 1995). At the LHF stage, *Pax2* is normally expressed as a transverse band corresponding to the presumptive mes-met region (Fig. 3I) (Rowitch and McMahon, 1995). At the four-somite stage, in addition to strong *Pax2* expression in the mes-met region, *Pax2* also is expressed in the anterior neural ridge and presumptive otic vesicles (Fig. 3M) (Hidalgo-Sanchez et al., 2001). In LHF stage $Otx2^{hOtx1/hOtx1}$ mutants, expression of *Pax2* was reduced and expanded anteriorly (Fig. 3J). By the four-somite stage, *Pax2* expression was restricted to the anterior tip of the neuroectoderm in $Otx2^{hOtx1/hOtx1}$ mutants, whereas its expression in the presumptive otic ectoderm appeared normal (Fig. 3N). In $Gbx2^{-/-}$ embryos, expression domain of *Pax2* was expanded caudally to the level of the expression in the otic ectoderm (Fig. 3K,O). Interestingly, in LHF stage $Gbx2^{-/-}$; $Otx2^{hOtx1/hOtx1}$ embryos, *Pax2* expression domain was expanded both anteriorly and posteriorly at the LHF (Fig. 3L), and by the four-somite stage its expression was detected throughout a broad region of the anterior neuroectoderm (Fig. 3P).

En1 is another early marker for the mes-met region and is first detected by the one-somite stage (Joyner et al., 2000). Interestingly, alteration of *En1* expression pattern mirrored that of *Pax2* expression in the mes-met domain of $Gbx2^{-/-}$, $Otx2^{hOtx1/hOtx1}$ and $Gbx2^{-/-}$; $Otx2^{hOtx1/hOtx1}$ mutant embryos. At the three-somite stage, *En1* expression was reduced and its expression domain was restricted to the anterior tip of $Otx2^{hOtx1/hOtx1}$ embryos (Fig. 3R). In $Gbx2^{-/-}$ embryos, the expression domain of *En1* was expanded caudally (Fig. 3S). By contrast, in $Gbx2^{-/-}$; $Otx2^{hOtx1/hOtx1}$ embryos, expression of *En1* was found in a broad region of the anterior neuroectoderm (Fig. 3T). In summary, in $Gbx2^{-/-}$; $Otx2^{hOtx1/hOtx1}$ embryos the expression of mes and met genes is abnormally colocalized in a broad anterior domain, suggesting that the presumptive midbrain and r1 regions are not differentially specified and instead a broad anterior region of neuroectoderm takes on characteristic of both regions. By contrast, in $Otx2^{hOtx1/hOtx1}$ embryos, the initial anterior expansion of the expression domains of *Gbx2* and then *Fgf8*, and a lack of initiation of *Wnt1* expression in the presumptive

mesencephalon, suggest that the mesencephalon is not specified normally and that the tissue is instead transformed into a metencephalic fate (see Fig. 8A).

A failure of differential specification of the mes- and met-regions in *Gbx2*$^{-/-}$; *Otx2*$^{hOtx1/hOtx1}$ embryos was further supported by expression of human *OTX1* from the *Otx2* locus. At E8.5, human *OTX1* is expressed in the forebrain and midbrain in *Otx2*$^{+/hOtx1}$ embryos (Fig. 3U), whereas its expression is absent in the anterior neuroectoderm of *Otx2*$^{hOtx1/hOtx1}$ embryos (Fig. 3V) (Acampora et al., 1998). In *Gbx2*$^{-/-}$; *Otx2*$^{hOtx1/hOtx1}$ embryos at the nine-somite stage, however, human *OTX1* was found in a broad anterior region, largely co-localized with *Wnt1*, *Fgf8*, *Pax2* and *En1* (Fig. 3W). Together, these results demonstrate that *Gbx2* and *Otx2* are dispensable for specification of a mes-met region. However, *Gbx2* and *Otx2* are essential for defining the spatial expression patterns of mes-met genes.

Gbx2$^{-/-}$; *Otx2*$^{hOtx1/hOtx1}$ embryos have an anterior deletion and exencephaly

Given the requirement for an interaction between *Otx2* and *Gbx2* in maintaining their early respective expression domains, some of the phenotypes seen in *Otx2* or *Gbx2* homozygous mutants could result primarily from the abnormal expansion of the *Gbx2* or *Otx2* expression domains, respectively. Indeed, the initial gene expression analysis above showed that expression of some midbrain markers, *Wnt1* and human *OTX1*, were restored in a broad anterior region of the neuroectoderm by removing *Gbx2* from *Otx2*$^{hOtx1/hOtx1}$ embryos at early somite stages. However, this anterior region appeared abnormally specified and composed of molecular attributes of both the mesencephalic and metencephalic regions. Furthermore, there were no consistent morphological differences between *Otx2*$^{hOtx1/hOtx1}$ and *Gbx2*$^{-/-}$; *Otx2*$^{hOtx1/hOtx1}$ embryos at these stages (Fig. 3, compare second and fourth rows), and both

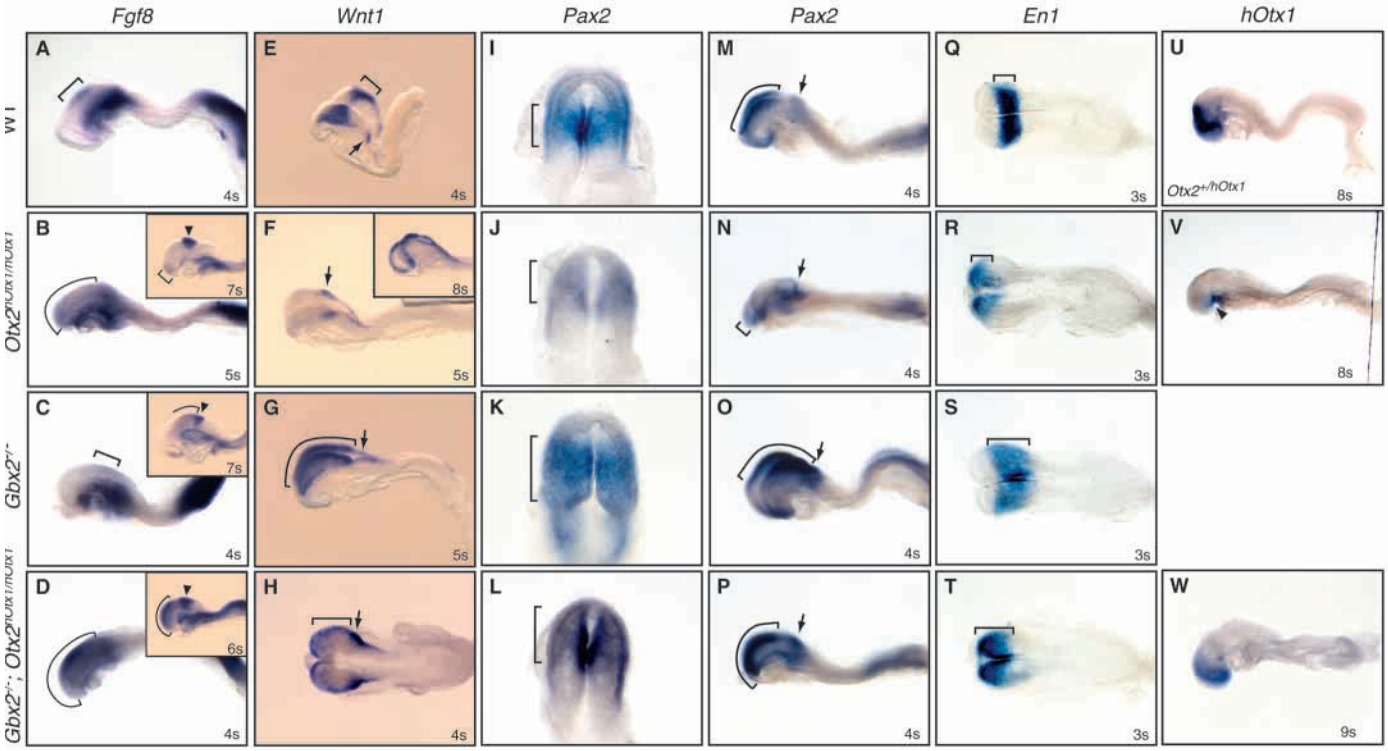

Fig. 3. Expression of mes-met genes in single *Otx2* or *Gbx2* and double homozygous mutant embryos at early somite stages. (A,B) Expression of *Fgf8* at the four-somite stage. Insets in the images show expression of *Hoxb1* (arrowheads) and *Fgf8* (brackets) in six- to seven-somite stage embryos. Note that in the *Gbx2*$^{-/-}$ embryo (inset in C), broad and weak *Fgf8* expression forms a gradient in the anterior hindbrain with highest expression overlapping with *Hoxb1* expression in r4. By contrast, *Fgf8* is expressed broadly in the anterior neuroectoderm of *Gbx2*$^{-/-}$; *Otx2*$^{hOtx1/hOtx1}$ embryos (inset in D) and its posterior limit ends a few cell diameters from *Hoxb1* expression in r4. (E-H) Expression of *Wnt1* in the posterior hindbrain (arrows) remains unchanged in embryos that lack *Otx2* (F), *Gbx2* (G) or both (H) at the four- to five-somite stage. The transverse band of *Wnt1* expression in the mesencephalon (brackets), however, is affected in these embryos. Inset in F shows an *Otx2*$^{hOtx1/hOtx1}$ embryos at the eight-somite stage with *Wnt1* expression in the lateral edges of the neural plate extending from the posterior hindbrain to the anterior extreme of the embryo. (I-L) and (M-P) Expression of *Pax2* at the LHF stage and four-somite stage, respectively, in embryos lacking *Otx2* (J,N), *Gbx2* (K,O) or both genes (L,P). The mes-met expression of *Pax2* is indicated by a bracket. Expression of *Pax2* in the pre-otic ectoderm is marked by arrows. (Q-T) *En1* expression (brackets) is shifted anteriorly in embryos that lack *Otx2* (R,T), whereas *En1* expression is expanded posteriorly in embryos that lack *Gbx2* (S,T). The *En1* expression level in *Otx2*$^{hOtx1/hOtx1}$ embryos is also reduced. (U-W) Human *OTX1* is expressed from the *Otx2* locus in *Otx2* heterozygous (U), *Otx2*$^{hOtx1/hOtx1}$ (V) and *Gbx2*$^{-/-}$; *Otx2*$^{hOtx1/hOtx1}$ (W) embryos. Human *OTX1* is expressed only in the anteriormost endoderm and ectoderm (arrowhead), but not in the neuroectoderm of *Otx2*$^{hOtx1/hOtx1}$ embryos, whereas in *Gbx2*$^{-/-}$; *Otx2*$^{hOtx1/hOtx1}$ embryos human *OTX1* is expressed in a broad anterior domain of the neuroectoderm. Note that all the *Otx2*$^{hOtx1/hOtx1}$ and *Gbx2*$^{-/-}$; *Otx2*$^{hOtx1/hOtx1}$ embryos have a similar anterior truncation (compare embryos in the second column with those in the forth column). The number of somites in the embryos is indicated in the lower right-hand corner of each panel. Anterior is towards the left, except for I-L,Q-T, which are dorsal views of embryos with anterior to the top.

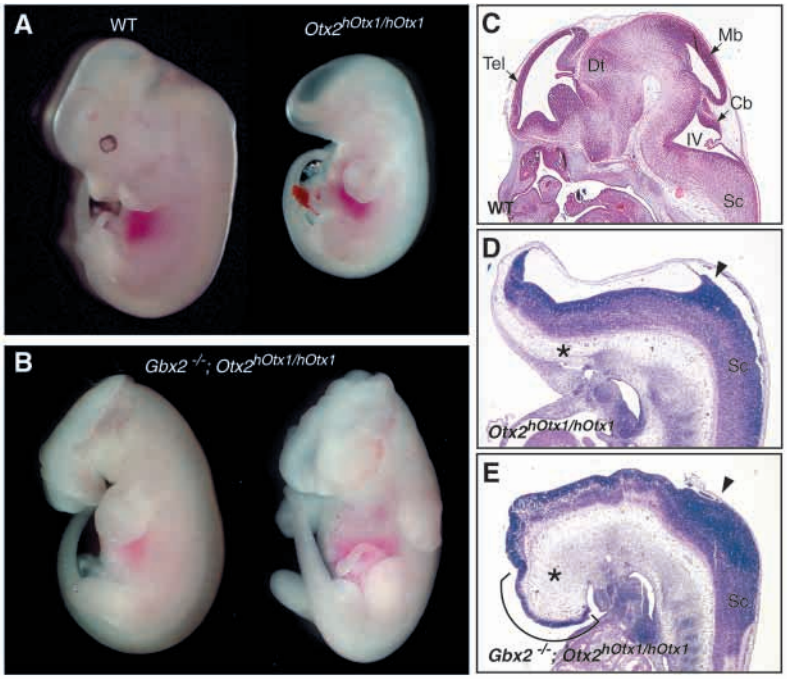

Fig. 4. *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos have an anterior truncation and exencephaly. (A,B) Morphology of wild-type (left in A), *Otx2*$^{hOtx1/hOtx1}$ (right in A) and *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos (B) at E12.5. (C-E) Sagittal sections of wild-type (C), *Otx2*$^{hOtx1/hOtx1}$ (D) and *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos (F) at E12.5. The anterior structures of *Otx2*$^{hOtx1/hOtx1}$ and *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos are largely truncated. There is more anterior tissue, particularly head mesenchyme (asterisk) in *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos than in *Otx2*$^{hOtx1/hOtx1}$ embryos. There is an additional thin epithelium (bracket) extending from the presumptive hindbrain in *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos. D,E are at a higher magnification than C. The junction between the spinal cord and hindbrain is marked by arrowheads. Cb, cerebellum; Dt, dorsal thalamus; IV, IVth ventricle; Mb, midbrain; Sc, spinal cord; Tel, telecephalon.

mutants had a similar anterior truncation. Interestingly, by E9.5, the morphology of the anterior structures of these two types of mutant embryos was significantly different. The anterior neural tube of *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos failed to close and the anterior neural fold was expanded laterally, whereas in *Otx2*$^{hOtx1/hOtx1}$ embryos, the anteriorly truncated neural tube consisted of a thin neural epithelium (data not shown). The majority of embryos deficient for *Otx2* died around E10.5. Remarkably, we did manage to recover an *Otx2*$^{hOtx1/hOtx1}$ and two *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos at E12.5 from a single litter (out of a total of 10 litters). At this stage, the most anterior structure of the *Otx2*$^{hOtx1/hOtx1}$ embryo was reminiscent of an anterior hindbrain, consisting of the IVth ventricle and the roof of the ventricle (Fig. 4A) (Acampora et al., 1998). Exencephaly persisted in the *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos at E12.5, and no discernable craniofacial or eye structures developed in these mutants (Fig. 4B). The *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos, however, appeared to have more anterior tissue compared with the *Otx2*$^{hOtx1/hOtx1}$ mutant. Histological analysis showed that in the *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos there was significantly more head mesenchyme and an additional thin layer of undifferentiated neuroectoderm at the anterior end of the brain, compared with the *Otx2*$^{hOtx1/hOtx1}$ embryo (Fig. 4D,E). Taken together, although there was some expansion of anterior tissue by removing *Gbx2* in *Otx2*$^{hOtx1/hOtx1}$ mutants, development of head structures was greatly impaired, and no discernable midbrain or cerebellar anlage developed in *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ mutants.

To characterize the regional identity of the anterior neural plate of *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos, we examined expression of markers that are distinctive for specific brain regions at E9.5. In wild-type embryos, *Wnt1* and *Fgf8* are expressed in two juxtaposed bands of cells at the mid-hindbrain junction, with *Wnt1* in the midbrain and *Fgf8* in the hindbrain, whereas *En1* is expressed broadly across both regions (Fig. 5A,C,E). *Otx2* is normally expressed in the forebrain and midbrain (Fig. 5G). In *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos, the expression of *Wnt1*, *Fgf8*, *En1* and human *OTX1* was seen to persist in a broad domain of the anterior neuroectoderm of E9.5 double mutant (Fig. 5B,D,F,H). By contrast, expression of *Fgf8* and *En1* was restricted to the anterior tip of *Otx2*$^{hOtx1/hOtx1}$ embryos and human *OTX1* was not detected in the neuroectoderm at this stage (data not shown) (Acampora et al., 1998). Thus, the anterior neural plate of *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos continues to have molecular characteristics of both midbrain and r1 at E9.5.

Normally, the respective expression limits of *Fgf8*, *Pax2*, *En1* and *En2* are at successively more posterior positions in r1 (Fig. 6A) (Joyner et al., 2000). To determine whether a similar spatial relationship is established in *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos, the posterior borders of expression of *Wnt1*, *Fgf8*, *En1*, *En2* and *Pax2* was analyzed on adjacent sagittal sections of a *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryo at E10.5. *Wnt1*, *Fgf8* and *Pax2* were co-expressed in a broad anterior neuroectoderm domain with a similar posterior limit (Fig. 6B-D). Expression of *En1* and *En2* encompassed the *Wnt1*, *Fgf8* and *Pax2* expression domains but the posterior *En1* and *En2* expression limits were extended caudally, with *En2* being more posterior (Fig. 6E,F). These results show that a normal spatial relationship of the posterior expression domains of *Fgf8*, *Pax2*, *En1* and *En2* is maintained in *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos.

Taken together, removal of *Gbx2* in *Otx2*$^{hOtx1/hOtx1}$ mutant embryos allows some early midbrain genes to be expressed, but does not rescue midbrain development. Persistent broad expression of *Fgf8*, overlapping with other mes-met genes in the anterior neuroectoderm may disrupt neural tube closure and normal differentiation of the midbrain and hindbrain.

The forebrain fails to develop in *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos

The abnormal expansion of mes-met genes to the anterior extreme of *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos at early somite stages indicated that the forebrain failed to develop in these mutants. To verify this, we examined expression of *Six3*, a homeobox gene that is normally expressed in the forebrain (Fig. 7A) (Oliver et al., 1995). Previous studies have shown that *Otx2* activity in the anterior neuroectoderm is not essential for

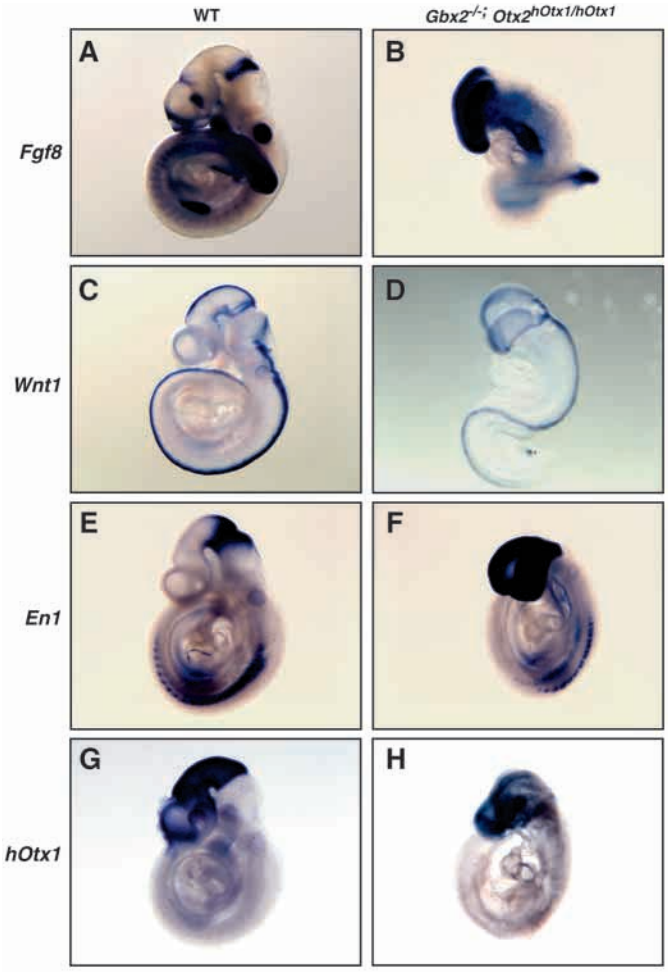

Fig. 5. Mes-met genes are maintained and co-expressed in the anterior neuroectoderm of *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos at E9.5. (A-H) Expression of *Fgf8* (A,B), *Wnt1* (C,D), *En1* (E,F) and human *OTX1* (G,H) in wild type (left column) and *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos (right column) at E9.5. *Fgf8* and *En1* are strongly expressed in a broad anterior region of the double mutant embryos and their expression appears to co-localize with that of *Wnt1* and human *OTX1*.

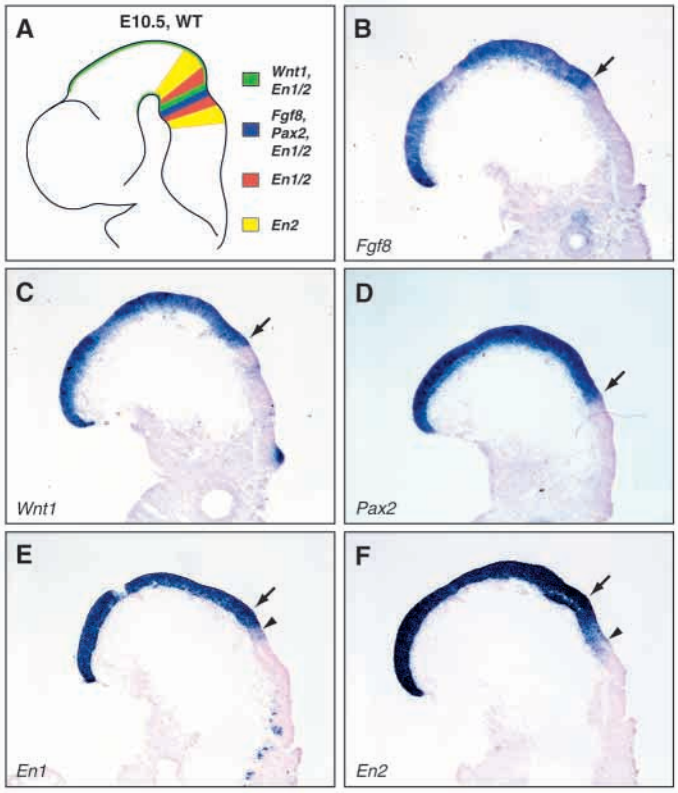

Fig. 6. Spatial relationship of the expression domains of mes-met genes in *Otx2*$^{hOtx1/hOtx1}$ embryos at E10.5. (A) The normal expression patterns of *Wnt1*, *Fgf8*, *Pax2*, *En1* and *En2* in the mid/hindbrain region at E10.5 (Joyner et al., 2000). (B-F) In situ hybridization analysis of expression of *Fgf8* (B), *Wnt1* (C), *Pax2* (D), *En1* (E) and *En2* (F) on near adjacent sagittal sections of a *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryo. Note that the posterior limits (arrows) of the expression domains of *Wnt1*, *Fgf8* and *Pax2* are similar, whereas the expression domains of *En1* and *En2* encompass those of *Wnt1*, *Fgf8* and *Pax2* and their posterior limits (arrowheads) are successively extended more caudally, with a decreasing gradient.

initiation of *Six3* expression but is required for maintenance of *Six3* expression (Acampora et al., 1998; Kimura et al., 2000). In agreement with these observations, *Six3* expression was detected in the anterior headfold of *Otx2*$^{hOtx1/hOtx1}$ and *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos at the LHF stage (data not shown). By the three-somite stage, expression of *Six3* in the anterior neuroectoderm was not detected in either *Otx2*$^{hOtx1/hOtx1}$ (n=2) or *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos (n=2) (Fig. 7C,D). As expected, in *Gbx2*$^{-/-}$ embryos *Six3* expression was normal (Fig. 7B). Furthermore, expression of *Bf1* (n=2) and *Hesx1* (n=1), two other forebrain markers (Thomas and Beddington, 1996; Shimamura and Rubenstein, 1997), was absent in the neuroectoderm of the double homozygous mutants at E8.5 (data not shown). Thus, removal of *Gbx2* is not sufficient to allow initiation of forebrain development in *Otx2*$^{hOtx1/hOtx1}$ embryos.

Development of r3, but not r2, is rescued in *Gbx2*$^{-/-}$ embryos by removing *Otx2* function

We have shown that *Otx2* expression is expanded posteriorly in *Gbx2*$^{-/-}$ embryos and *Krox20* expression is not initiated in r3, reflecting a loss of specification of r1-3 (see Fig. 1). To investigate whether removal of *Otx2* in *Gbx2*$^{-/-}$ embryos rescues hindbrain development, we examined gene expression in r2 and r3. Expression of *Krox20* was examined in embryos at the eight-somite stage. In *Gbx2*$^{-/-}$ embryos, only a single stripe of cells weakly expressing *Krox20* was detected in r5 (Fig. 7B) (Wassarman et al., 1997). Strikingly, in *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos, two transverse stripes of *Krox20* expression were seen, similar to that in wild-type and *Otx2*$^{hOtx1/hOtx1}$ embryos (Fig. 7A,C,D), suggesting r3 is rescued in *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos.

We next examined the expression of *Hoxa2*, which normally is strongly expressed in r3, r5 and weakly in r2 at E9.5 (Fig. 7E). The neural crest cells migrating out from r4 also express *Hoxa2*. In *Gbx2*$^{-/-}$ embryos, *Hoxa2* expression in r5 and the migrating neural crest cells from r4 appeared normal, but there was no *Hoxa2* expression in r2-3 (Fig. 6F). This result further supports our previous studies showing that the defects in *Gbx2* mutants are limited to r1-3. As expected, *Hoxa2* expression in *Otx2*$^{hOtx1/hOtx1}$ embryos was essentially normal, although the

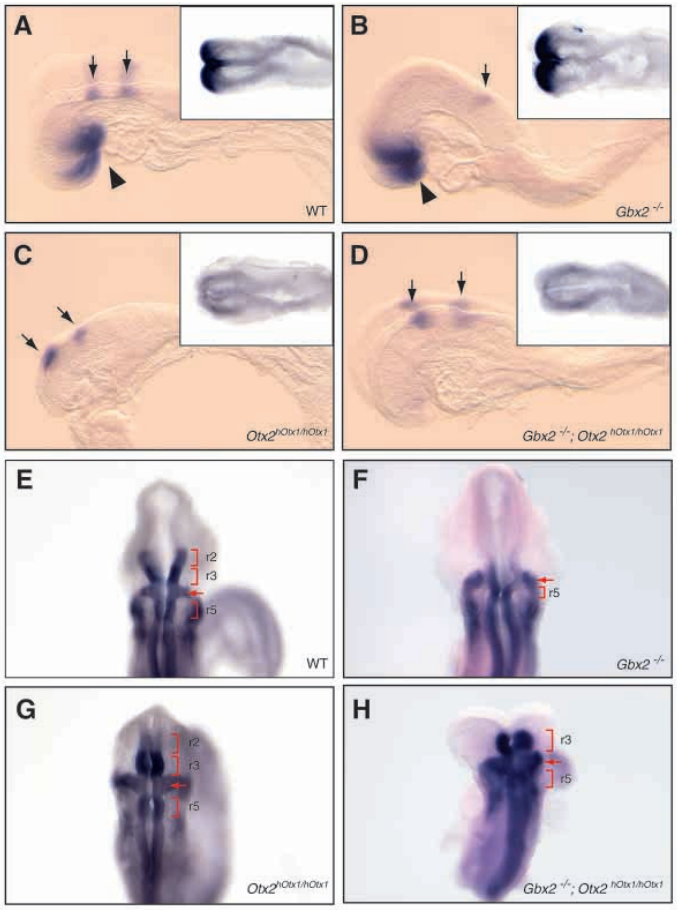

Fig. 7. Expression of *Krox20* in r3 is restored in *Gbx2* mutants by removing *Otx2* function. (A-D) Double labeling for *Six3* (arrowheads) and *Krox20* (arrows) expression in wild-type (A), *Gbx2*$^{-/-}$ (B), *Otx2*$^{hOtx1/hOtx1}$ (C) and *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ (D) embryos at the eight-somite stage. Insets show *Six3* expression in embryos of corresponding genotypes at the 3-somite stage. Two stripes of *Krox20* expressing cells (arrows), corresponding to r3 and r5, are found in wild type, *Otx2*$^{hOtx1/hOtx1}$ and *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos, but there is only a single stripe of *Krox20* expression in r5 of *Gbx2*$^{-/-}$ embryos. (E-H) *Hoxa2* expression in wild-type (E), *Gbx2*$^{-/-}$ (F), *Otx2*$^{hOtx1/hOtx1}$ (G) and *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ (H) embryos at E9.5. *Hoxa2* expression in rhombomeres is bracketed, whereas the expression in neural crest cells migrating from r4 is indicated by arrows. R3 expression of *Hoxa2* is rescued in *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ mutant embryos, whereas r2 expression of *Hoxa2* is missing.

expression domains in r2 and r3 appeared slightly expanded (Fig. 7G). Interestingly, in *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos *Hoxa2* expression was detected in r3, as well as in r5 and the neural crest cells migrating from r4 (Fig. 7H). In these mutants, expression of *Hoxa2* in r3 was slightly expanded as in *Otx2*$^{hOtx1/hOtx1}$ embryos. Significantly, expression of *Hoxa2* was not detected in r2 of *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos. Taken together, these results show that development of r3, but not r2, was rescued in *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos. Therefore, *Gbx2* is not directly involved in initiating r3 formation, but instead is required to limit *Otx2* expression to the anterior neuroectoderm and thus allow r3 development to proceed.

DISCUSSION

In this study, we have focused on the phenotypes of *Otx2* and *Gbx2* single and double homozygous mutants at early developmental stages. We show that the initial expression domains of *Otx2* and *Gbx2* are established independently, although their expression rapidly becomes interdependent. We further show that although RA can regulate *Otx2* negatively and *Gbx2* positively, the repression of *Otx2* by RA requires *Gbx2*. We demonstrate that *Otx2* and *Gbx2* are not essential for the initiation of mes-met gene expression. Furthermore, although *Fgf8* and other mes-met genes are induced in *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos, the expression domains of these genes are abnormally co-localized in a broad anterior region of the neuroectoderm (summarized in Fig. 8A), uncovering negative regulatory roles of *Gbx2* and *Otx2* in midbrain and hindbrain development, respectively. Consistent with this, removal of *Otx2* from *Gbx2* homozygous mutant embryos rescues segmental development of r3, demonstrating that *Gbx2* plays only a permissive role in r3 development by limiting *Otx2* expression to the anterior neuroectoderm. By contrast, the forebrain fails to develop in *Gbx2*$^{-/-}$*; Otx2*$^{hOtx1/hOtx1}$ embryos, but midbrain gene expression is restored. Therefore, the abnormal anterior expansion of *Gbx2* is responsible for the loss of midbrain but not forebrain development in *Otx2* mutants.

Establishment of the expression domains of *Otx2* and *Gbx2*

During gastrulation, expression of *Otx2* and *Gbx2* is dynamic and the expression domains of these two genes become complementary. Interestingly, the initial expression patterns of *Gbx2* or *Otx2* are generally preserved in embryos deficient in *Otx2* or *Gbx2*, respectively, although a reciprocal antagonistic interaction between *Otx2* and *Gbx2* is crucial in maintaining their expression limits at the presumptive mes-met border as early as the LHF stage. In agreement with these results, the expression domains of *Otx2* and *Gbx2* do not immediately abut each other at the EHF stage in mouse embryos (J. Y. H. L. and A. L. J., unpublished) and similar observations were recently made in the chick (Garda et al., 2001). Therefore, the initial *Otx2-Gbx2* border must be established by external factors. Previous studies have shown that the expression domain of *Otx2* is defined by both positive and negative signals (Ang et al., 1994). One possible scenario is that *Otx2* and *Gbx2* are regulated by the same pathways, but with opposite responses. RA has been considered a candidate for such a signal. Indeed, previous studies and the work presented here have shown that RA can repress *Otx2* and induce *Gbx2*. However, in this study, we demonstrate that repression of *Otx2* by RA is dependent on *Gbx2*. Therefore, RA cannot play an essential role in regulating the initial expression of *Otx2* in vivo.

Other posteriorizing factors, like Fgfs, are plausible regulators of early *Otx2* and *Gbx2* expression. Multiple Fgfs, including *Fgf3*, *Fgf4*, *Fgf5*, *Fgf8* and *Fgf17* (Sun et al., 1999), are expressed in posterior regions of the mouse embryo during gastrulation. Furthermore, Fgf8-soaked beads can induce *Gbx2* and repress *Otx2* in the midbrain of chick embryos or in embryonic mouse brain explants (Irving and Mason, 1999; Liu and Joyner, 2001; Liu et al., 1999; Martinez et al., 1999; Garda et al., 2001). Significantly, unlike RA, Fgf8 can repress *Otx2* independent of *Gbx2* function (Liu and Joyner, 2001). Finally,

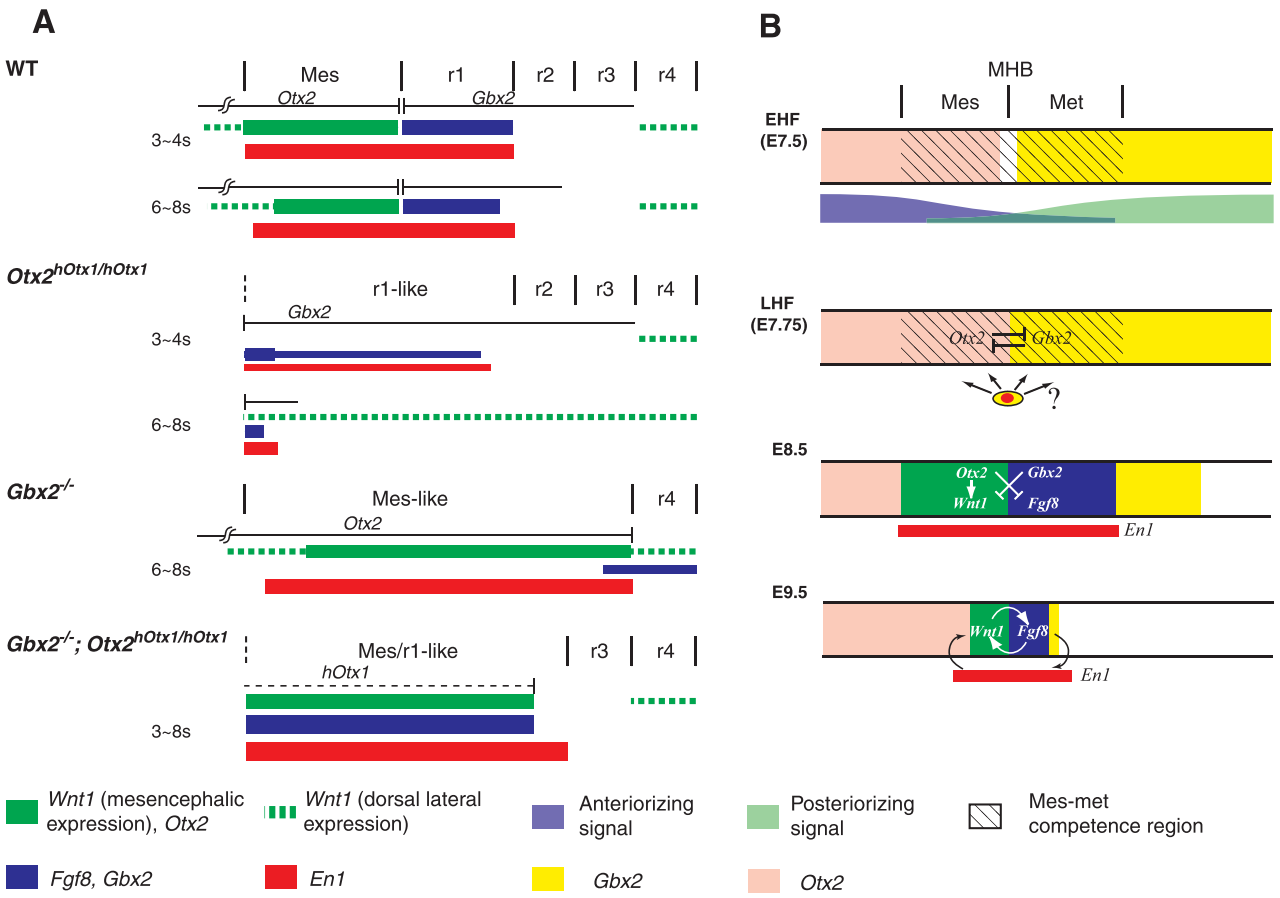

Fig. 8. Establishment of the spatial relationships of mes-met genes at the *Otx2-Gbx2* border. (A) Expression of *Wnt1*, *Fgf8* and *En1* in wild-type, *Otx2*$^{hOtx1/hOtx1}$, *Gbx2*$^{-/-}$ and *Gbx2*$^{-/-}$; *Otx2*$^{hOtx1/hOtx1}$ embryos at the three- to four-somite and six- to eight-somite stages. Thickness of the bars represents the level of gene expression. (B) Model of how the mes-met genes are induced and how the normal spatial expression of these genes is established in the neuroectoderm. Opposing interactions between posteriorizing and anteriorizing signals determine the position of the *Otx2/Gbx2* border, as well as a mes-met competence domain by E7.5. The entire presumptive mes-met region is competent to respond to a signal that induces expression of *Wnt1*, *Fgf8* and other mes-met genes. Expression of *Otx2* and *Gbx2* in the neuroectoderm at E7.75 subdivides the presumptive mes-met region into two distinct domains. Negative regulation of *Wnt1* by Gbx2, and of *Fgf8* by Otx2 results in the initial restriction of *Wnt1* and *Fgf8* expression specifically to the *Gbx2*- and *Otx2*-negative positive regions, respectively, at E8.5. At later stages, expression of *Wnt1* and *Fgf8* is maintained only in cells adjacent to each other through mutual positive feedback between *Fgf8* and *Wnt1* (or an unknown secreted factor in the midbrain) and between all mes-met genes. *Otx2* and *Gbx2* continue to negatively regulate *Fgf8* and *Wnt1* expression, respectively. MHB, mid-hindbrain boundary.

in *Fgf8*-null mutant embryos, which also fail to express *Fgf4* in the primitive streak, *Otx2* is expressed throughout the epiblast, whereas *Gbx2* is not expressed (Sun et al., 1999). These results strongly implicate Fgfs in regulating the initial expression of *Otx2* and *Gbx2*.

How is the mes-met genetic cascade initiated?

One surprising result of our study is that expression of *Fgf8* and other mes-met genes is initiated and maintained in the absence of both *Gbx2* and *Otx2*. Based on analysis of the genetic mechanisms that regulate formation of local organizers in various developmental systems, Meinhardt proposed that an interaction between differentially specified fields leads to expression of secreted factors in cells at the common border of the two fields (Meinhardt, 1983). Supporting this hypothesis, two recent transplantation studies in chick embryos demonstrated that juxtaposition of r1 tissue and posterior forebrain or midbrain tissue is sufficient to induce *Fgf8* at a new *Otx2-Gbx2* border (Irving and Mason, 1999; Hidalgo-Sanchez et al., 1999b). Furthermore, Garda et al. (Garda et al., 2001) observed a transient overlap in expression of *Otx2* and *Gbx2* that proceeded mes-met expression of *Fgf8* and argued that this interaction between *Otx2* and *Gbx2* is essential for the induction of *Fgf8*. Contradictory to these models, we show in this study that although the mesencephalic and metencephalic regions fail to be differentially specified in embryos lacking both *Otx2* and *Gbx2*, *Fgf8* and other mes-met genes are induced and maintained (Fig. 8A).

The finding that *Fgf8* and *Wnt1* are induced in a broad domain in *Gbx2*$^{-/-}$; *Otx2*$^{hOtx1/hOtx1}$ embryos indicates that the presumptive mesencephalon and metencephalon are equally competent to express *Fgf8* and *Wnt1*, as well as other mes-met genes, in response to an inductive signal. One role of *Otx2* and *Gbx2* is therefore to restrict *Fgf8* and *Wnt1* expression to their appropriate regions through negative regulation. A key question now is how a mes-met competence domain is initially

specified. Gene expression studies have indicated that specification of the mes-met domain occurs as the neuroectoderm induced. For example, *Pax2* is initially expressed in the anterior region of mouse embryos as early as the late streak stage and becomes restricted to a transverse band marking the presumptive mes-met region by the LHF stage (Rowitch and McMahon, 1995). Furthermore, explants of anterior ectoderm from late streak stage mouse embryos express *En1* after 2 days in culture, suggesting that induction of *En1* is already autonomous to the anterior ectoderm by the late streak stage (Ang and Rossant, 1993). A similar observation was recently made in chick (Muhr et al., 1999).

The initial regionalization of the neural plate is thought to be achieved by two opposing signals: posteriorizing signals released from the node and molecules expressed by the anterior visceral endoderm and mesendoderm that antagonize the posteriorizing signals (Stern, 2001). These two opposing signals may determine the position of the *Otx2*-*Gbx2* border, as well specifying a mes-met competence domain (Fig. 8B). This hypothesis is supported by the fact that mouse mutations in genes that function in the anterior visceral endoderm or/and anterior mesendoderm (*Otx2*, *Lim1*, *Nodal* and *Smad2*) disrupt development of the mes-met region, as well as formation of more anterior regions (Beddington and Robertson, 1998). Furthermore, it has been shown that both inductive signals derived from the anterior mesendoderm and posterior mesendoderm determine the expression domain of *Otx2* (Ang et al., 1994), as well as *En1/2* (Muhr et al., 1999). Finally, we have shown in this study that the prospective *Otx2*-*Gbx2* border, as well as a mes-met competence domain is initially defined independent of *Otx2* and *Gbx2*.

The molecular identity of the signal(s) inducing the mes-met cascade is still unknown. Because after initiation of the mes-met cascade, it can probably be maintained by *Fgf8* and intricate mutual regulation among the mes-met genes, we predict that the initial inductive signal is transient. Genetic studies have so far failed to identify the initial inductive signal for the mes-met cascade. Fgf4- or Fgf8-soaked beads inserted into the diencephalon or anterior midbrain of chick embryos at the 10-somite stage can initiate a de novo induction of mes-met genes including *Fgf8*, suggesting Fgf4 and Fgf8 may mimic the normal induction mechanism of the mes-met cascade (Shamim and Mason, 1998; Crossley et al., 1996; Martinez et al., 1999). Paradoxically, Fgf8-soaked beads can not induce *Fgf8* or *Pax2* in E9.5 mouse brain tissue explants (Liu et al., 1999; Garda et al., 2001). It is not clear, however, whether the neuroectoderm from E9.5 embryos has the same competence as E8.0 embryos, when the mes-met genes are initially induced. Previous studies have shown that Fgf signaling is required for the initiation and maintenance of *En1/2* expression in chick epiblast explants (Muhr et al., 1999). Interestingly, *Spry2*, a likely downstream target of Fgf signaling, is expressed specifically in the presumptive mes-met region at the LHF stage (J. Y. H. L. and A. L. J., unpublished), indicating the presence of Fgf signaling in this region when mes-met gene expression is initiated. Similarly, activated extracellular signal-related kinases, which mediate signaling of various receptor tyrosine kinases including Fgfs, were detected specifically in the presumptive mid-hindbrain boundary and the primitive streak of *Xenopus* embryos at stage 12.5, a stage before mes-met gene initiation (Christen and Slack, 1999). These observations indicate that the Fgf signaling pathway could be involved in induction of the mes-met cascade.

The location of a mes-met induction signal(s) is also elusive. *Fgf4* is expressed in the notochord underlying the presumptive mes-met region in chick embryos and this expression has been implicated to be important for initiation of the mes-met cascade (Shamin et al., 1999). Recently, it has been shown that *Fgf18* is transiently expressed in the chick head process (Ohuchi et al., 2000). Similar expression of *Fgf4* and *Fgf18* has not been reported in other species, and the functional significance of these gene expression has not been tested. Several groups have investigated possible source of mes-met inductive signals using in vitro explant culture, in which the naïve neuroectoderm is co-cultured with different potential inductive tissues or signaling molecules. It has been shown that the anterior mesendoderm is sufficient to induce expression of both *Otx2* and *En1/2* in pre-streak stage anterior and posterior ectoderm (Ang and Rossant, 1993; Ang et al., 1994). In agreement with this, surgical removal of anterior midline mesendoderm and ectoderm from E7.5 mouse embryos in culture disrupts initiation of *Fgf8* expression in the mes-met region (Camrus et al., 2000). Furthermore, Muhr and colleagues have demonstrated that a rostralizing signal from the anterior mesendoderm, together with Fgfs, and an unknown signal from the paraxial mesoderm are essential for induction of En expression in chick epiblast explants (Muhr et al., 1999). Finally, in terms of later differentiation, neuroectoderm explants have been used, and it has been shown that neuronal differentiation with characteristics of the mid-hindbrain region resulted from an interaction between Fgf8, which is locally expressed in the mid-hindbrain junction, or Fgf4, which is expressed in the primitive streak, with Shh, which is expressed in the floor plate (Ye et al., 1998). These studies indicate that induction of the mes-met cascade may result from convergence of multiple signaling pathways.

Otx2 and *Gbx2* are required for establishment of the normal spatial relationships of mes-met genes

We have shown that in embryos lacking both *Otx2* and *Gbx2*, expression of mes-met genes, such as *En1*, *Pax2*, *Fgf8* and *Wnt1*, is initiated at similar stages to those in wild-type embryos and maintained at strong levels until at least to E10.5. However, in such mutants, expression of the mes-met genes is co-localized in a broad domain of the anterior neural plate (Fig. 8A). The entire neuroectoderm anterior to r3 displays molecular markers of both mes and met regions, including human *OTX1* (expressed from the *Otx2* locus), *Fgf8* and *Wnt1*. Thus, in mouse embryos that lack both *Otx2* and *Gbx2* the mes-met region develops as a single unit. *Otx2* and *Gbx2*, therefore, act as selector genes to subdivide the prospective mes-met region into two distinct domains.

Previous studies have indicated that *Otx2* regulates *Wnt1* positively (Rhinn et al., 1999) and *Fgf8* negatively (Acampora et al., 1997). Conversely, *Gbx2* appears to regulate *Wnt1* negatively and possibly *Fgf8* positively (Millet et al., 1999; Liu and Joyner, 2001; Katahira et al., 2000). We show that the initial expression domains of *Fgf8* and *Wnt1* are broad and overlapping in $Gbx2^{-/-}$; $Otx2^{hOtx1/hOtx1}$ embryos, demonstrating a crucial role for *Gbx2* and *Otx2* in an initial restriction of *Wnt1* and *Fgf8* to *Gbx2*- and *Otx2*-negative domains, respectively (Fig. 8B). Interestingly, expression of

Fgf8 is greatly reduced in *Otx2*$^{hOtx1/hOtx1}$ embryos at early somite stages, whereas the level of *Fgf8* expression in *Gbx2*$^{-/-}$; *Otx2*$^{hOtx1/hOtx1}$ embryos is initially comparable with that in wild-type embryos and later becomes upregulated. These results indicate that factors other than *Otx2* and *Gbx2* must be involved in modulating *Fgf8* expression.

Normally, the expression domains of *Wnt1* and *Fgf8* become progressively restricted to two sharp transverse rings immediately next to each other at the mid-hindbrain border by E9.5, after their initial broad and complementary expression at E8.5. Concurrently, the expression domains of *Pax2* and *En1* become restricted to bands that straddle the mid-hindbrain border. The molecular basis that underlies this compaction of gene expression remains to be elucidated. Recently, it was shown that Fgf8 can induce *Wnt1* expression and maintain *Wnt1* expression only in *Gbx2*-negative cells (Liu and Joyner, 2001). In a complementary manner, *Wnt1* may also positively regulate *Fgf8*, as expression of *Fgf8* is rapidly lost in the metencephalon of mouse embryos deficient in *Wnt1* (Lee et al., 1997). Consistent with this, previous studies have also implicated a secreted factor from the mesencephalon in positive regulation of *Fgf8* (Irving and Mason, 1999; Danielian and McMahon, 1996). It is possible that once *Wnt1* and *Fgf8* are induced in two adjacent domains, only cells close to their common border maintain *Wnt1* and *Fgf8* expression through cell-nonautonomous actions of Fgf8 and Wnt1 (or an unknown factor), respectively, and thus their expression domains become restricted to the boundary region (Fig. 8B). The progressive restriction of *Fgf8* and *Wnt1* may also account for the later restriction of *Pax2*, *En1* and *En2* to this region, as a synergy between *Fgf8* and *Wnt1* appears to be responsible for maintaining their expression (Danielian and McMahon, 1996; Crossley et al., 1996). Based on this, it is likely that in *Gbx2*$^{-/-}$; *Otx2*$^{hOtx1/hOtx1}$ embryos, a failure in segregation of the *Fgf8*- and *Wnt1*-expressing domains prevents the normal compaction of the *Fgf8* and *Wnt1* expression domains. Additionally, positive regulatory interactions among the mes-met genes likely contribute to the high levels of mes-met gene expression in *Gbx2*$^{-/-}$; *Otx2*$^{hOtx1/hOtx1}$ mutants. It is interesting to note that *Wnt1* is an exception among the mes-met genes in that expression of *Wnt1* is not upregulated in *Gbx2*$^{-/-}$; *Otx2*$^{hOtx1/hOtx1}$ embryos at late somite stages. This is in agreement with genetic evidence that *Otx2* plays a positive regulatory role in *Wnt1* expression (Rhinn et al., 1999).

In conclusion, we demonstrate that a mes-met region is specified independently of *Otx2* and *Gbx2*. However, subdivision of the mes-met region into two distinct units requires *Otx2* and *Gbx2*. Furthermore, *Otx2* and *Gbx2* primarily play negative regulatory roles in establishing the normal spatial expression domains of mes-met genes, with Otx2 repressing *Fgf8* and Gbx2 repressing *Wnt1*. Juxtaposition of *Wnt1* and *Fgf8* expression at the mes-met border is probably a prerequisite for establishment of a normal mid-hindbrain organizer.

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