EN and GBX2 play essential roles downstream of FGF8 in patterning the mouse mid/hindbrain region

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SUMMARY

Fgf8, which is expressed at the embryonic mid/hindbrain junction, is required for and sufficient to induce the formation of midbrain and cerebellar structures. To address through what genetic pathways FGF8 acts, we examined the epistatic relationships of mid/hindbrain genes that respond to FGF8, using a novel mouse brain explant culture system. We found that En2 and Gbx2 are the first genes to be induced by FGF8 in wild-type E9.5 diencephalic and midbrain explants treated with FGF8-soaked beads. By examining gene expression in En1/2 double mutant mouse embryos, we found that Fgf8, Wnt1 and Pax5 do not require the En genes for initiation of expression, but do for their maintenance, and Pax6 expression is expanded caudally into the midbrain in the absence of EN function. Since E9.5 En1/2 double mutants lack the mid/hindbrain region, forebrain mutant explants were treated with FGF8 and, significantly, the EN transcription factors were found to be required for induction of Pax5. Thus, FGF8-regulated expression of Pax5 is dependent on EN proteins, and a factor other than FGF8 could be involved in initiating normal Pax5 expression in the mesencephalon/metencephalon. The En genes also play an important, but not absolute, role in repression of Pax6 in forebrain explants by FGF8. Previous Gbx2 gain-of-function studies have shown that misexpression of Gbx2 in the midbrain can lead to repression of Otx2. However, in the absence of Gbx2, FGF8 can nevertheless repress Otx2 expression in midbrain explants. In contrast, Wnt1 is initially broadly induced in Gbx2 mutant explants, as in wild-type explants, but not subsequently repressed in cells near FGF8 that normally express Gbx2. Thus GBX2 acts upstream of, or parallel to, FGF8 in repressing Otx2, and acts downstream of FGF8 in repression of Wnt1. This is the first such epistatic study performed in mouse that combines gain-of-function and loss-of-function approaches to reveal aspects of mouse gene regulation in the mesencephalon/metencephalon that have been difficult to address using either approach alone.

Key words: Engrailed, Lmx1b, Wnt1, Gbx2, Otx2, Pax5, Pax6, Fibroblast growth factor, Organizer, Mouse

INTRODUCTION

Fgf8, which is expressed at the junction between the midbrain (mesencephalon/metencephalon) and anterior hindbrain (mesencephalon or met), has been shown in both chick and mouse to have an organizing activity that can induce ectopic expression of many mes/met genes and direct ectopic midbrain and cerebellar (anterior hindbrain) development in the posterior forebrain or midbrain (Crossley et al., 1996; Liu et al., 1999; Martinez et al., 1999; Shamim et al., 1999). Mes/met junction, or isthmic, tissue has a similar activity in heterotopic transplantation studies (reviewed by Alvarado-Mallart, 1993; Wassef and Joyner, 1997). Partial loss-of-function studies in mouse and fish support the idea that Fgf8 is also essential for mes/met development (Brand et al., 1996; Meyers et al., 1998; Reifers et al., 1998). However, how FGF8 signaling is transmitted, and through what genetic pathways it acts, still remain to be determined.

In addition to Fgf8, Wnt1, En1/2 and Pax2/5 are expressed early in the mes/met region, with Wnt1 expressed in the mes in a band of cells anterior to Fgf8, and En1/2 and Pax2/5 in mes and met cells surrounding the isthmus (reviewed by Wassef and Joyner, 1997; Joyner et al., 2000). Loss-of-function studies in both mouse and zebrafish have demonstrated that these families of genes are also required for early development of the mes/met region (reviewed by Wassef and Joyner, 1997; Joyner et al., 2000). Furthermore, gain-of-function studies have shown that mis-expression of En1/2 or Pax2/5 in chick or fish posterior forebrain results in ectopic expression of mes/met genes including Fgf8, and later induction of mes/met development (Araki and Nakamura, 1999; Funahashi et al., 1999; Okafuji et al., 1999; Ristoratore et al., 1999).
Gbx2 and Otx2 are the first genes known to be expressed in a restricted manner in domains of the mes/met. Their complementary patterns of expression in the anterior or posterior brain with a common border near the mes/met organizer suggested they have antagonistic roles in normal patterning of the midbrain and cerebellum (Wassef and Joyner, 1997; Joyner et al., 2000). Indeed, mouse mutants with no Otx2 expression in the epiblast fail to maintain rostral neural tissues, including the forebrain and midbrain, whereas ectopic expression of Otx2 in the anterior hindbrain of mouse and chick embryos results in repression of Gbx2 in the met, posterior expansion of the midbrain and partial deletion of the cerebellum (Acampora et al., 1998; Rhinn et al., 1998; Broccoli et al., 1999; Katahira et al., 2000). In a complementary manner, in Gbx2 null mutants, anterior hindbrain tissue is lost and there is a posterior expansion of the Otx2 expression domain and midbrain tissue (Wassarman et al., 1997; Millet et al., 1999). Furthermore, ectopic expression of Gbx2 in the posterior midbrain of mouse or chick embryos results in repression of Otx2 and a rostral shift of the isthmic expression domains of Fgf8 and Wnt1, leading (in mouse embryos) to a transient reduction of the midbrain and expansion of the hindbrain at E9.5 (Millet et al., 1999; Katahira et al., 2000). These genetic studies show that a reciprocal negative interaction between Gbx2- and Otx2-expressing cells is indeed critical for mes/met patterning through positioning the mes/met border and maintaining a normal organizer.

Epistasis analysis in which gain- and loss-of-function mutants are combined has been extensively performed in many invertebrate species and has proven to be a powerful tool for determining the order of gene action during embryonic development. Owing to technical limitations, epistasis studies have been difficult to perform in vertebrates, although one study that was performed provided evidence that En1 is a downstream target of Wnt1 (Damielian and McMahon, 1996). A simple system that allows for quick epistasis studies in the mouse would be of great value in unraveling the molecular network underlying the formation and function of a normal mid/hindbrain organizer.

We recently described a mouse brain explant culture system that allows for a direct examination of the epistatic relationships between genes that respond to FGF8 (Liu et al., 1999). Using wild-type brain explants, we previously showed that FGF8 can induce En1, En2 and Pax5 in E9.5 diencephalic explants, Gbx2 in both midbrain and diencephalic explants and repress Otx2 in midbrain explants by 40 hours. In addition, FGF8 induces Wnt1 in a ring of cells several cell diameters away from the FGF8 bead. In the current study, we found that FGF8 can also induce Lmx1b in midbrain explants and repress Pax6 in posterior forebrain explants. Furthermore, En2 and Gbx2 are the first genes to be induced by FGF8 within 8 hours, and alterations in expression of Pax5, Wnt1, Otx2 and Pax6 do not occur until 16-40 hours. We extended these studies by determining the epistatic relationships of genes downstream of FGF8 signaling using explants taken from different mutant and transgenic embryos and examining gene expression in early En1/2 mutant embryos. Diencephalic explant assays using mutant explants showed that the two En genes are required for induction of Pax5 by FGF8, and in turn PAX5 can upregulate expression of an En2 mid/hindbrain enhancer that contains PAX2/5-binding sites. Furthermore, while the En genes are not required for regulation of Gbx2, Wnt1 or Otx2 by FGF8, they are involved in, but not absolutely required for, repression of Pax6 in diencephalic explants. In contrast, we found that in En1/2 double mutant embryos, Fgf8, Wnt1 and Pax5 expression is initiated at early somite stages, but lost or greatly reduced by the 11-somite stage and Pax6 expands into the midbrain. The changes in expression of Pax5 and Pax6 in En1/2 mutants could be due to the early decrease in Fgf8 expression and a factor other than FGF8 could be responsible for inducing the initial Pax5 expression. Finally, although Gbx2 is not required for the induction of Wnt1, Lmx1b or En2 and the repression of Otx2 by FGF8, it is indeed required for the exclusion of Wnt1-expressing cells from around a FGF8 source in midbrain explants. These studies place En and GBX2 downstream of FGF8 in regulating Pax5/6 and Wnt1 expression, respectively, and GBX2 upstream of, and/or parallel to, FGF8 in regulating Otx2.

MATERIALS AND METHODS

Breeding and genotyping of the mutant embryos

Both En1/2 and Gbx2 mutants were kept on a mixed genetic background between 129 and Swiss Webster. En1/2 mutants were generated by Southern blot hybridization (Millet et al., 1994; Hanks et al., 1995). Gbx2 mutants were genotyped using a PCR approach (Wassarman et al., 1997). En1<sup>+/−</sup>; En2<sup>+/−</sup> F<sub>2</sub> males were crossed to either En1<sup>−/−</sup>; En2<sup>−/−</sup> or En1<sup>−/−</sup>; En2<sup>−/−</sup> females to obtain En1/2 double homozygous mutant embryos. Gbx2<sup>−/−</sup> mice were intercrossed to produce Gbx2 homozygous mutant embryos.

Generation of transgenic animals

The En2-CX and En2-ACX transgenic lines were generated as described by Song et al. (Song et al., 1996). Transgenic animals were genotyped by a PCR reaction using lacZ-specific primers (Liu et al., 1999) and homozygotes were genotyped by comparing the intensities of the Southern blot hybridization signals obtained using a lacZ-specific probe and digestion of tail DNA with EcoRI, with the ones obtained using an En2 3′-probe that detects the endogenous gene (Millet et al., 1994).

Explant cultures, X-gal staining and whole-mount RNA in situ hybridization

Explant cultures were carried out as described previously (Liu et al., 1999) except that the concentration of FGF8b solution was 0.2 mg/ml, unless otherwise indicated. X-gal staining and whole-mount RNA in situ hybridization were performed as described in Liu et al. (Liu et al., 1999). The antisense riboprobes used for RNA in situ hybridization were performed as described in Liu et al. (Liu et al., 1999) except that the concentration of FGF8b solution was 0.2 mg/ml, unless otherwise indicated. X-gal staining and whole-mount RNA in situ hybridization were performed as described in Liu et al. (Liu et al., 1999). The antisense riboprobes used for RNA in situ hybridization analyses were prepared using previously published mouse sequences, En1, En2 (Millet et al., 1995), Fgf8 (Crossley and Martin, 1995), Gbx2 (Bouillet et al., 1995), Lmx1b (Chen et al., 1998), Pax5 (Asano and Gruss, 1992), Pax6 (Grindley et al., 1997), Otx2 (Simeone et al., 1993) and Wnt1 (Parr et al., 1993).

RESULTS

Gbx2 and En2 are the first genes to be induced by FGF8

As a first step in dissecting the genetic pathway downstream of FGF8 signaling during mes/met development, the temporal order of gene expression alterations was compared in E9.5 wild type diencephalic explants (En1, En2, Pax5, Wnt1, Pax6 and Gbx2 expression), or midbrain explants (En2, Pax5, Wnt1,
downregulated in En1/2 double mutants at early many mes/met genes are initiated but quickly Gbx2 the roles for En and En1/2 double mutants used En2 earlier than expression of other genes makes it possible that En2 Lmx1b et al., 1999), whereas Lmx1b expression was more restricted than seen at 16 hours, in cells adjacent to the beads (Fig. 1D). The fact that En2 and Gbx2 expression was altered by FGF8 earlier than expression of other genes makes it possible that En2 and Gbx2 could be in higher tiers than the other genes in the genetic hierarchy of FGF8 signaling. We therefore used transgenic and mutant mouse brain explants to further examine the roles for En and Gbx2 in the FGF8 signaling pathway.

Many mes/met genes are initiated but quickly downregulated in En1/2 double mutants at early somite stages

Previous studies of En1 (Wurst et al., 1994) and En2 (Millen et al., 1994) single mutants and an allele in which En1 was replaced with En2 (Hanks et al., 1995; Hanks et al., 1998) demonstrated that the two genes have overlapping functions. In order to determine the normal requirement for both En genes during early patterning of the mouse mes/met region, before expression of other genes makes it possible that En2 and Gbx2 could be in higher tiers than the other genes in the genetic hierarchy of FGF8 signaling. We therefore used transgenic and mutant mouse brain explants to further examine the roles for En and Gbx2 in the FGF8 signaling pathway.

| Table 1. Gene expression profiles in brain explants cultured with FGF8b-soaked beads |
|---------------------------------|-----|-----|-----|-----|-----|-----|-----|
|                                | En1 | En2 | En2 | Pax5 | Pax6 | Gbx2 | Otx2 | Wnt1 |
|                                | i   | i   | i   | r   | r   | i   | r   | i   |
| (A) Diencephalic explants      |     |     |     |     |     |     |     |     |
| 8 hours                        | 0/2 | 2/3§| nd  | nd  | nd  | nd  | nd  | nd  |
| 16 hours                       | 2/3§| 3/3§| 0/3 | 1/2*| 1/2*| 0/3 | 1/2*| 0/3 |
| 40 hours                       | 2/5 | 2/5 | 2/5 | 2/5 | 2/5 | 2/5 | 2/5 | 2/5 |
| (B) Midbrain explants          |     |     |     |     |     |     |     |     |
| 8 hours                        | 2/2§| 2/2§| 2/2§| nd  | nd  | nd  | nd  | nd  |
| 16 hours                       | 2/2§| 2/2§| 2/2§| nd  | nd  | nd  | nd  | nd  |
| 40 hours                       | 2/2§| 2/2§| 2/2§| nd  | nd  | nd  | nd  | nd  |

*Incomplete repression was seen.
†In BSA- and FGF8b-treated explants, Otx2 was variably partially lost.
§At these stages, expression was weak and in a limited domain of cells
i, induced; nd, not determined; r, repressed.
was weaker, with expression of Pax5 and Fgf8 found in more restricted domains than in their En1+/−; En2+/− littermates (Fig. 3B, B′, C, C′, D, D′, E, E′). By the 10-12 somite stage, no major morphological deletion of tissue could be detected in En1/2 double homozygous mutants compared with their En1+/−; En2+/− littermates (compare Fig. 3F with 3F′). Indeed, lacZ expression from the En1 locus in double En1/2 mutants was in a similar mes/met domain to in En1+/−; En2+/− littermates (Fig. 3F, F′). In contrast to En1-lacZ expression in En1/2 double homozygous mutants, Pax5 (n=2) and Fgf8 (n=2) expression was much weaker and in more restricted domains compared with their littermates (Fig. 3H, H′, J, J′). Furthermore, Fgf8 expression was not seen in one out of three double mutants examined at this stage (data not shown). Wnt1 expression in the transverse band seen in the posterior midbrain of En1+/−; En2+/− embryos (Fig. 3I) was not seen in double homozygous 11-somite mutants (Fig. 3I′ n=2). Furthermore, in the double homozygous mutants, the lateral expression of Wnt1 was continuous along the anteroposterior axis, unlike in En1+/−; En2−/− and wild-type embryos, in which Wnt1 expression along the lateral edge of the neural plate was absent in the anterior hindbrain. Gbx2 expression in r1 was also much weaker in En1/2 double homozygous mutant compared with their En1+/−; En2+/− littermates (Fig. 2G, G′).

In chick embryos, the expression domain of the En genes abuts that of the diencephalon gene Pax6 during early mes/met development. Furthermore, misexpression of En1 in the diencephalon leads to repression of Pax6 (Araki and Nakamura, 1999). Based on these observations it was suggested that the En genes are involved in setting up the forebrain/midbrain boundary by repressing the forebrain gene Pax6. In mouse, the En1 mes/met expression domain abuts the Pax6 forebrain expression domain briefly before the eight-somite stage (A. L. and A. L. J., unpublished observations). To determine whether the En genes are required to maintain the normal Pax6 expression pattern, En1/2 early somite double homozygotes were analyzed for Pax6 expression. In 11-somite En1/2 mutants, Pax6 expression was expanded posteriorly compared with that in En1+/−; En2+/− littermates, but the expansion was seen only in the lateral (dorsal) part of the neural plate and formed a decreasing gradient posteriorly, suggesting that EN proteins are involved in, but not the only factors required for, repressing Pax6 in the mes/met region (Fig. 3K, K′).

**EN proteins are required for induction of Pax5 and involved in the repression of Pax6 by FGF8**

In En1/2 double homozygous mutant E9.5 embryos, the constriction between the telencephalon and dorsal diencephalon is apparent, and a minor constriction can be seen posterior to Otx2 and Pax6 expression domains (arrowheads in Fig. 2C′, D′). Based on our gene expression studies we assume that the region between the two constrictions corresponds to the diencephalon in En1/2 mutants. Tissue in the anterior two-thirds of this diencephalic region was taken for explant culture to ensure that no hindbrain tissue was included. Such explants were compared with diencephalic explants taken from apparently normal En1+/−; En2−/− littermates. En1-lacZ expression was induced in explants taken from double homozygous mutant embryos, or from their wild-type-appearing littermates after 40 hours in culture with FGF8b-soaked beads, showing that EN proteins are not required for induction of transcription from the En1 locus by FGF8b (Fig. 4A-C). We subsequently examined Pax5 mRNA expression in the same explants. Pax5 induction was robust in explants taken from En1+/−; En2+/− and En1−/−; En2+/− embryos (Fig. 4A and data not shown). In contrast, Pax5 induction was not detected in the double En1/2 homozygous mutant embryos cultured with beads soaked in either 0.2 mg/ml (Fig. 4C, n=2) or 1mg/ml (inset in Fig. 4C, n=3) FGF8b protein solution. Interestingly, Pax5 expression was barely detected in only one out of three En1+/−; En2+/− explants and not detected in the other two (Fig. 4B and data not shown). These studies demonstrate that unlike in En1/2 mutant embryos at early somite stages where Pax5 is not dependent on EN function, the En genes are required for induction of Pax5 by FGF8. Furthermore, En2, and not En1, is the limiting factor downstream of FGF8 in the process of activating Pax5 in forebrain explants.

We next examined whether the EN proteins are required for repression of Pax6 by FGF8 in diencephalic explants. As described above, when forebrain explants were taken from wild-type embryos and cultured for 40 hours, Pax6 expression was greatly reduced in cells adjacent to FGF8b-soaked beads (Fig. 4D). In contrast to wild-type explants, Pax6 was variably
and only partially repressed by FGF8 in *En1/2* double homozygous mutant explants (n=4). In one explant, the PAX6-negative region was more restricted to cells in the vicinity of the beads than in wild type explants (Fig. 4E). In the other three explants, two of which were cultured with beads soaked in 1 mg/ml FGF8b protein solution, cells adjacent to the beads showed weak *Pax6* expression (Fig. 4F). These results show that the En genes are involved in the repression of *Pax6* by FGF8 in the forebrain, but that other factors must also be involved.

We also examined the induction of Gbx2 (n=2) and Wnt1 (n=2) expression in *En1/2* double homozygous mutant explants treated for 40 hours with FGF8b-soaked beads; the two genes showed similar responses to their *En1/2+/−; En2/−* litters and wild-type embryos (data not shown). This shows that FGF8 can regulate *Gbx2* and *Wnt1* via EN-independent pathways.

**PAX2/5-binding sites are required for the upregulation of an *En2* mes/met reporter by FGF8**

We have previously shown that two PAX2/5-binding sites within a 1 kb *En2* mes/met enhancer fragment (*En2-CX*) are required for expression of a *lacZ* reporter gene in the region of the mes/met junction in early mouse embryos (Song et al., 1996), indicating that PAX2/5 are involved in regulation of *En2* in the mes/met, in a reciprocal manner to the EN regulation of *En1* expression in *Fgf8* embryos. The embryos in A-E are *En1+/−; En2−* embryos and the ones in A’-E' are *En1+/−; En2+/−* embryos. In *En1+/−; En2−* embryos, a loss of mes/met tissue is morphologically obvious and Fg8 and *Pax5* expression in the mes/met region is missing. The caudal boundary of the *Pax6* forebrain expression domain is coincident with the caudal boundary of the *En2* PBD-*lacZ* expression (Fig. 4F). These results show that the En genes are involved in the repression of *Pax6* by FGF8 in the forebrain, but that other factors must also be involved.

*Gbx2* is not required for repression of *Otx2* by FGF8 but is required for late repression of *Wnt1* in cells close to FGF8-soaked beads

We have previously shown that when midbrain explants are cultured with FGF8b-soaked beads, *Gbx2* is induced by 40 hours in *Otx2*-negative cells, and that there is also a ring of *Gbx2*- and *Otx2*-negative cells surrounding the *Gbx2*-expressing cells (Liu et al., 1999). In addition, *Wnt1*-expressing cells are induced adjacent to the *Gbx2*-expressing cells. This spatial relationship of gene expression, and the fact that *Gbx2* is induced before the alterations in *Otx2* and *Wnt1* expression, suggests that *Gbx2* could play a direct role in regulating *Otx2* and *Wnt1* expression by FGF8. Since *Fg8* mes/met expression is abnormal in *Gbx2* mutant embryos (Wassarman et al., 1997;
Millet et al., 1999), it is not possible to distinguish in the mutants whether the deregulation of Otx2 and Wnt1 is due to the abnormal Fgf8 expression or whether GBX2 is directly required for regulating their expression. We sought to address this question by using midbrain explants taken from Gbx2 mutants.

Gbx2-/- embryos have a deletion of the anterior hindbrain and an expansion of the midbrain at E9.5, whereas the diencephalon appears normal, based on both morphological landmarks and Pax6 staining (Wassarman et al., 1997 and data not shown). Explants were taken from the anterior half of the midbrain of Gbx2 mutants and wild-type E9.5 embryos. After 40 hours in culture with FGF8, Otx2 expression was completely repressed in cells adjacent to the beads in all wild-type explants (Fig. 6A, n=8) or Gbx2 heterozygotes (not shown, n=13). Similarly, Otx2 was completely repressed in all Gbx2 homozygous explants by FGF8 (Fig. 6B; n=10). This result shows that a Gbx2-independent pathway exists for mediating repression of Otx2 by FGF8, and that the caudal expansion of Otx2 expression into the Fgf8 expression domain in Gbx2 mutant embryos could result at least in part from compromised Fgf8 expression.

We have shown that in wild-type forebrain and midbrain explants cultured with FGF8b-soaked beads, Wnt1 expression is consistently found in a ring of cells that are several cell diameters away from the beads after 40 hours (Liu et al., 1999; Fig. 6C). This pattern was also observed in Gbx2 heterozygous explants treated with FGF8b-soaked beads (not shown, n=3). However, in midbrain explants from Gbx2 homozygous mutants, strong Wnt1 expression was detected in the cells right adjacent to the beads after 40 hours, showing that Gbx2 is required for the late repression of Wnt1 in cells near a source of FGF8.
Essential roles of EN and GBX2 in FGF8 signaling

DISCUSSION

In this study we explored some of the epistatic relationships between mes/met genes using mouse explants from En and Gbx2 mutants since these are the first genes to be induced by FGF8. Using En mutant explants we show that both En genes are required for FGF8 induction of Pax5, but that they are not the only genes required for repression of Pax6 in diencephalic tissue (Fig. 7A). Since in En1/2 double homozygous mutant embryos we found that all the mes/met genes examined, including Pax5, are initially expressed, a factor(s) other than FGF8 is likely to be responsible for inducing Pax5 mes/met expression. The downregulation of Pax5 expression by the 11-somite stage in En mutants, however, could indicate that Fgf8 and/or the En genes play a later role in maintaining Pax5 transcription. Using Gbx2 mutant explants we determined that Gbx2 plays a specific role in excluding Wnt1 expression in cells near an FGF8 source. This role of Gbx2 could account for the normal exclusion of Wnt1 from cells in the metencephalon and expansion of the Wnt1 expression domain into the metencephalon in Gbx2 mutants. Consistent with this, we observed a downregulation of Gbx2 expression and ectopic Wnt1 expression in the metencephalon of 11-somite En1/2 mutant embryos. Surprisingly, although previous experiments showed that Gbx2 misexpression in the mesencephalon is sufficient to lead to repression of Otx2 (Millet et al., 1999; Katahira et al., 2000), in our explant system Gbx2 was not required for a complete repression of Otx2 by FGF8b. These studies represent one of the first epistasis studies carried out in mice, or other vertebrates, and have uncovered a new level of complexity in the genetic hierarchy of genes downstream of FGF8 that regulate mes/met anteroposterior patterning.

Pax2/5 and En1/2 are involved in a feedback loop

Previously we showed that two Pax2/5-binding sites are required for the mid/hindbrain expression of an En2-lacZ reporter gene, suggesting PAX2/5 might be involved in direct regulation of En2 (Song et al., 1996). However, further studies showed that transcription of the reporter is much more restricted to the mes/met junction region than the endogenous En2 gene, indicating that there are other critical DNA regulatory sequences in the En2 locus (Song and Joyner, 2000). Indeed, deletion of these Pax2/5-binding sites from the endogenous En2 gene does not abolish En2 expression, but only decreases its initial expression (Song and Joyner, 2000). We found that the same Pax2/5-binding sites are required for upregulation of the En2-lacZ transgene by FGF8 in forebrain explants and the timing of the upregulation is consistent with Pax5 first being induced and then Pax5 inducing the transgene. In contrast, the endogenous En2 gene is induced before Pax5 in forebrain explants by FGF8, and Pax2 and Pax8 are not induced at all (Table 1; Liu et al., 1999). Therefore, the normal early induction of En2 expression by FGF8 must be through a Pax2/5-independent pathway.

More interestingly, Pax5 is not induced in diencephalic explants by FGF8 in the absence of the En genes, showing that EN proteins could be involved normally in regulating Pax5. Taken together with the transgenic studies, this indicates that a positive feedback loop exists between En2 and Pax5. It has been found that misexpression of En1/2 in chick diencephalic tissue first results in repression of Pax6 expression and then in induction of Pax5 expression (Araki and Nakamura, 1999). Consistent with the En misexpression studies, we found in our diencephalic explants, that Pax6 was partially repressed by 16 hours, whereas Pax5 was not induced until 40 hours. Furthermore, since Pax6 was not fully repressed in En1/2 double mutant diencephalic explants, it is possible that this accounts for the lack of induction of Pax5 by FGF8 in such mutants. The fact that Pax5 expression is initiated in En1/2...
mutant embryos could mean that Pax5 is regulated not only by FGF8 and EN, but also by other proteins, consistent with multiple transcription regulator-binding sites being present in Pax5 cis-regulatory sequences (Pfeffer et al., 2000). Alternatively, Pax5 can be induced only by FGF8 in En1/2 mutants in tissue that does not express Pax6. Ectopic expression of En genes not only results in repression of Pax6 and induction of Pax5 in the diencephalon but also development of midbrain structures in both chick and medaka fish (Araki and Nakamura, 1999; Ristoratore et al., 1999). Furthermore, since expression of an activator form of EN2 in the chick midbrain inhibits midbrain development and results in upregulation of Pax6, the primary function of EN2 is likely to be a repressor (Araki and Nakamura, 1999). It has been suggested that a negative feedback loop between En genes and Pax6 establishes the midbrain/forebrain border (Araki and Nakamura, 1999). We found that in mouse, unlike in chick, the En1 mes/met expression domain normally only abuts the Pax6 forebrain expression domain briefly at early somite stages (data

expression of En genes not only results in repression of Pax6 and induction of Pax5 in the diencephalon but also development of midbrain structures in both chick and medaka fish (Araki and Nakamura, 1999; Ristoratore et al., 1999). Furthermore, since expression of an activator form of EN2 in the chick midbrain inhibits midbrain development and results in upregulation of Pax6, the primary function of EN2 is likely to be a repressor (Araki and Nakamura, 1999). It has been suggested that a negative feedback loop between En genes and Pax6 establishes the midbrain/forebrain border (Araki and Nakamura, 1999). We found that in mouse, unlike in chick, the En1 mes/met expression domain normally only abuts the Pax6 forebrain expression domain briefly at early somite stages (data

Fig. 5. PAX2/5 DNA-binding sites are essential for upregulation of an En2-lacZ reporter in forebrain explants in response to FGF8b. (A,B) Schematic showing the En2-lacZ transgene (A) consisting of a 1kb En2 enhancer (red) with two PAX2/5-binding sites (green ovals) that drives expression of a lacZ gene (blue) through a heat shock minimal promoter (black arrow), and the En2PBD-lacZ transgene (B) in which the PAX2/5-binding sites are deleted. (C) The En2-lacZ transgene is expressed in the mouse mes/met region at E9.5 (blue staining). (D) The En2PBD-lacZ transgene fails to be expressed in the mes/met region. (E,G) FGF8b can upregulate expression of the En2-lacZ reporter (red staining) in forebrain explants after 40 hours (E), but not after 16 hours (G). Inset in G shows an explant with BSA-soaked beads showing similar low-level lacZ expression to that in G. (F) The En2PBD-lacZ transgene is not upregulated by FGF8b in forebrain explants. C, Ccl; X, XbaI.

Fig. 6. GBX2 is required for the repression of Wnt1 but not Otx2 expression in midbrain explants treated with FGF8b. (A,B) Otx2 is repressed by FGF8b in both wild-type (A) and Gbx2<sup>-/-</sup> (B) anterior midbrain explants. (C,D) Wnt1 is repressed in cells adjacent to the beads by 40 hours in wild-type (C) but not in Gbx2<sup>-/-</sup> (D) midbrain explants, instead it is expressed in cells near the FGF8b-soaked beads. (E,F) Lmx1b is induced in cells adjacent to the FGF8b beads by 40 hours in wild-type midbrain explants (E). This induction is not altered in Gbx2<sup>-/-</sup> explants (F). (G,H) En2 is strongly induced in both wild-type (G) and Gbx2<sup>-/-</sup> (H) anterior midbrain explants by FGF8b-soaked beads but not by BSA-soaked beads (inset in H). Red arrowheads point to regions of strong Otx2 and Wnt1 expression. The positions of the beads are highlighted with green rings.
GBX2 is required for the repression of Wnt1 by FGF8b, but not for the repression of Otx2 and activation of Wnt1

Previous loss-of-function studies have shown that the expression domains of Otx2 and Wnt1 are expanded caudally in Gbx2 mutants from E8.5 onwards, suggesting that Gbx2 might be required to repress Otx2 and/or Wnt1 (Millet et al., 1999). Furthermore, gain-of-function studies have shown that misexpression of Fgf8 or Gbx2 is sufficient to lead to repression of Otx2 (Liu et al., 1999; Martinez et al., 1999; Millet et al., 1999; Katahira et al., 2000). In contrast, our explant studies showed that the expression domains of Gbx2 and Otx2 following FGF8 application are not directly adjacent to each other, but instead that cells expressing neither gene are induced between the two expression domains (Liu et al., 1999). As further direct proof that Gbx2 is not the only gene involved in repressing Otx2, we have shown that FGF8 can repress Otx2 in midbrain explants taken from Gbx2 mutant embryos. Our results indicate that either FGF8 induces two pathways that lead to Otx2 repression, only one of which is dependent on GBX2, or that GBX2 first upregulates Fgf8 in the Gbx2 misexpression experiments and this leads to repression of Otx2 (Fig. 7B). The latter is consistent with the finding that in Otx1/2 hypomorphic mutants, Fgf8 expression is first expanded rostrally and then Otx2 expression is repressed and expression of Gbx2 and Wnt1 induced anteriorly (Acampora et al., 1997). Therefore, it is possible that the deregulation of Otx2 expression in Gbx2 mutants is an indirect outcome of loss of Gbx2 function, possibly due to decreased expression of Fgf8, as was previously suggested (Wassarman et al., 1997).

In contrast, Gbx2 plays a more direct role in regulating expression of Wnt1. Normal regulation of Wnt1 expression is extremely important, since it is required for development of the entire mes/met region, as well as for stabilizing the mes/met boundary (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Thomas et al., 1991; McMahon et al., 1992; Bally-Cuif et al., 1995). After 40 hours in wild-type midbrain explants, Wnt1 is expressed in a ring of cells several cell diameters away from FGF8-soaked beads and Gbx2 is expressed in the Wnt1-negative cells close to the bead (Liu et al., 1999). We now show that this expression pattern is the result of two events. Initially, Wnt1 is induced in a broad region around the FGF8-soaked beads and then Wnt1 is only maintained in cells at a distance to the beads. The fact that Wnt1 expression expands caudally in Gbx2 mutants from E8.5 onwards (Millet et al., 1999), and our finding that in E9.5 Gbx2 mutant midbrain explants Wnt1 is induced and maintained in a broad region by FGF8, provide evidence that GBX2 is required for repression of Wnt1 in cells near an FGF8 source. Consistent with this, the ectopic expression of Wnt1 in the lateral edges of the mesencephalon in En1/2 mutants could be due to the decrease of Gbx2 expression. Our explant studies have revealed an interesting mechanism by which FGF8 regulates Wnt1 expression, first through activation and then repression, and that this two-step process involves different pathways, since only one is GBX2 dependent.

It was found that Otx2 is required in a cell-autonomous manner for the expression of Wnt1 at the mes/met junction, based on studies of mouse chimeras containing Otx2+/− and wild-type cells (Rhinn et al., 1999). From these studies it was not clear whether Otx2 regulates Wnt1 directly. Two results with our explant assays suggest that the regulation is likely indirect. First, in midbrain explants Wnt1 is induced by FGF8 in Otx2-negative cells (Liu et al., 1999). Second, we show here that in the absence of Gbx2, regulation of Wnt1 and Otx2 are dissociated. Otx2 can be repressed, but Wnt1 is nevertheless induced and maintained in Otx2-negative cells, showing that Otx2 is not required to directly regulate Wnt1 expression. Instead, Otx2 might normally allow Wnt1 to be expressed in the midbrain by repressing Gbx2 expression. In early somite embryos, Wnt1 expression normally progresses from broad mesencephalic expression to expression only at the midbrain/hindbrain junction. Furthermore, Wnt1 is upregulated in Otx2-positive midbrain cells surrounding Otx2 mutant cells in chimeras. It is therefore possible that Wnt1 expression can be regulated by a positive signal, and our explant studies indicate that FGF8 is a good candidate. It would therefore be very interesting to know whether the Otx2−/− cells in the midbrain of Otx2−/−→wild-type chimeras do express Gbx2 and Fgf8.

Lmx1b was recently implicated as a positive regulator of Wnt1 mes/met expression in chick (Adams et al., 2000). Consistent with this, we found that Lmx1b was induced by FGF8 several hours before Wnt1, and scattered expression of both genes was seen initially. By 40 hours, however, Wnt1 was
expressed only in Gbx2-negative cells at a distance from the beads, whereas Lmx1b was in both Gbx2-positive and -negative cells (Fig. 1 and data not shown). The latter is consistent with the fact that in chick the Lmx1b mes/met domain extends more posteriorly than that of Wnt1, overlapping with Fgf8 and Gbx2 expression (Adams et al., 2000). Based on our studies, it is possible that in the mes/met border during normal development, FGF8 regulates Wnt1 expression positively through Lmx1b and negatively through Gbx2, hence positioning Wnt1 expression anterior to the mes/met border, adjacent to, but not overlapping with, Fgf8.

In summary, by using brain explants taken from the posterior forebrain and anterior midbrain of mutants, we have obtained new insights into the epistatic relationships between different mes/met genes regulated by FGF8 signaling. Such information could not be gained from a direct analysis of the phenotypes of various mutants because of the simultaneous early alterations in expression of multiple genes and, in some cases, loss of tissue. This new information furthers our understanding of how FGF8 functions to pattern the midbrain and cerebellum along the anteroposterior axis, and to maintain a normal boundary between the midbrain and hindbrain. Finally, the studies have revealed that there are multiple pathways and additional factors involved in FGF8 signaling and organizer function that have yet to be identified.

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