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Sequential roles for *Otx2* in visceral endoderm and neuroectoderm for forebrain and midbrain induction and specification

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SUMMARY

The homeobox gene *Otx2* is a mouse cognate of the *Drosophila orthodenticle* gene, which is required for development of the brain, rostral to rhombomere three. We have investigated the mechanisms involved in this neural function and specifically the requirement for *Otx2* in the visceral endoderm and the neuroectoderm using chimeric analysis in mice and explant recombination assay. Analyses of chimeric embryos composed of more than 90% of *Otx2*$^{-/-}$ ES cells identified an essential function for *Otx2* in the visceral endoderm for induction of the forebrain and midbrain. The chimeric studies also demonstrated that an anterior neural plate can form without expressing *Otx2*. However, in the absence of *Otx2*, expression of important regulatory genes, such as *Hesx1/Rpx*, *Six3*, *Pax2*, *Wnt1* and *En*, fail to be initiated or maintained in the neural plate. Using explant-recombination assay, we could further demonstrate that *Otx2* is required in the neuroectodem for expression of En. Altogether, these results demonstrate that *Otx2* is first required in the visceral endoderm for the induction, and subsequently in the neuroectoderm for the specification of forebrain and midbrain territories.

Key words: *Otx2*, forebrain, midbrain, patterning, chimera, ES cell, explant, *orthodenticle*, *Drosophila*, mouse

INTRODUCTION

The molecular mechanisms that control regional specification of the vertebrate brain are largely unknown, although many genes homologous to *Drosophila* pattern-formation genes are expressed in the embryonic brain and are candidates for playing a role in its regionalization. Genetic analysis of head development in *Drosophila* has identified three gap genes, *orthodenticle*, *empty spiracles* and *buttonhead*, that are important for the development of head segments (reviewed in Cohen and Jurgens, 1991; Finkelstein and Perrimon, 1991). *Otx1*, *Otx2* (Simeone et al., 1993), and *Emx1* and *Emx2* (Simeone et al., 1992) are cognate genes of *otd* and *ems* genes, respectively, in mice. These genes show overlapping expression patterns in the forebrain and midbrain. Other genes such as *Six3* (Oliver et al., 1995), a homologue of *sine oculis*, *Pax2* (Rowitch and McMahon, 1995), a homologue of *paired*, and *En1* and *En2* (Davis and Joyner, 1988), homologues of *engrailed*, also show restricted expression domains in the forebrain, midbrain and/or anterior hindbrain of early mouse embryos. These genes encode paired domain or homeodomain proteins that are likely to be transcription factors. Their restricted expression patterns along the anteroposterior (A-P) axis of the neural tube suggest that they may control the stepwise regionalization of the brain along this axis.

Otx2 is the earliest homeobox-containing gene to be expressed in the neuroectoderm. *Otx2* is a *bicoid*-class homeobox gene, which has also been isolated from other species, including sea urchin (Gan et al., 1995), *Xenopus* (Pannese et al., 1995), chick (Bally-Cuif et al., 1995) and zebrafish (Li et al., 1994, Mercier et al., 1995). In mouse, *Otx2* is already expressed in the entire ectoderm and visceral endoderm before gastrulation at E5.5-6.0 (Simeone et al., 1993; Ang et al., 1994). As gastrulation proceeds (E6.0-E7.5), this expression becomes progressively restricted to the anterior end of the embryo in all three germ layers. In vitro tissue recombination experiments in mouse and *Xenopus* have demonstrated that interactions with axial mesendoderm tissues are involved in the progressive restriction of *Otx2* expression to the anterior end of the embryo (Ang et al., 1994; Blitz and Cho, 1995). In mice, this restriction starts as soon as the early gastrulation stage (E6.5), suggesting that visceral endoderm surrounding the epiblast at this stage could also be involved in this restriction.

To study the function of *Otx2* during mouse embryogenesis, we and others have generated mutations in *Otx2* by homologous recombination in ES cells (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). Homozygous mutant embryos show early gastrulation defects, in particular in the development of axial midline cells, and they fail to develop forebrain, midbrain and anterior hindbrain. However, these studies did not pinpoint the mechanism leading to the loss of

rostral brain tissue. In vivo transplantation and in vitro tissue recombination experiments, pioneered by Spemann and Mangold (reviewed in Lemaire and Kodjabachian, 1996), have demonstrated that an interaction between ectoderm and axial mesendoderm plays an important role in the formation and regionalization of the neural tube. Since *Otx2* is expressed in both the ectoderm and the axial mesendoderm at the anterior end of the embryo at the time of neural plate formation (Ang et al., 1994), it could be required in either of the two tissues for rostral brain development. *Otx2* could be required in mesendodermal cells for the production of signals inducing the anterior neural plate formation. Alternatively, loss of rostral brain tissue could be due to an autonomous function of *Otx2* in the ectoderm and/or neurectoderm.

A third possibility is that *Otx2* is required in the visceral endoderm for development of the rostral brain. Visceral endoderm derives from the primitive endoderm and, thus, has a different origin from embryonic endoderm that gives rise to the gut and is derived from the epiblast only later, at E6.5. During gastrulation, the visceral endoderm joins with the extraembryonic mesoderm to form the visceral yolk sac (VYS). The critical role of the VYS in the maternofetal exchange of nutrients has been well documented (Jollie, 1990). Visceral endoderm cells have also been shown in gene targeting experiments to play an important role in the growth of the epiblast (Chen et al., 1994; Spyropoulus et al., 1994; Duncan et al., 1997). In addition, recent embryological and chimeric studies in mice have provided evidence for a role of visceral endoderm in patterning the rostral brain during gastrulation (Thomas and Beddington, 1996; Varlet et al., 1997).

In this work, we aimed at better understanding the function of *Otx2* in anterior neural tube development, revealed by the loss of rostral brain tissue in $Otx2^{-/-}$ embryos. We first show that the anterior neural plate is missing in these embryos at the earliest stage identifiable. These results strongly suggest that the loss of rostral brain in $Otx2^{-/-}$ embryos is due to a lack of induction of the anterior neural plate, which normally requires ectoderm-mesendoderm interactions. To determine in which germ layers *Otx2* is required for the induction and regionalization of the rostral brain tissue, we performed chimera studies with homozygous $Otx2^{-/-}$ ES cells and in vitro tissue recombination experiments between $Otx2^{-/-}$ and wild-type embryos. Chimeric embryos composed of more than 90% $Otx2^{-/-}$ cells show a rescue of the anterior neural plate, due to the presence of wild-type cells in the visceral endoderm. This rescued rostral neural tissue, which consists entirely of $Otx2^{-/-}$ cells, however fails to express some forebrain and midbrain genes. In addition, $Otx2^{-/-}$ ectoderm did not express En1 and En2 (En) proteins after recombination with wild-type mesendoderm in vitro. Altogether, these results demonstrate that *Otx2* is required in the visceral endoderm for anterior neural plate induction, and subsequently in the neuroectoderm for regional specification of the forebrain and midbrain.

MATERIALS AND METHODS

Targeting vector

A 1 kb *Otx2* cDNA (Ang et al., 1994) was used to isolate from a 129Sv/J genomic library two overlapping genomic clones, g3 and g1 (Fig. 1A), encoding the entire coding region of the *Otx2* gene. The *Not*I-*Not*I insert of genomic clone, g3 was subcloned into pBluescriptIIKS (+) (Stratagene), to generate a plasmid called BSg3. The g3-hygro targeting vector was constructed by replacing the 4 kb *Bgl*II fragment of plasmid BSg3 with a 2 kb *Cla*I-*Xho*I fragment containing a PGKhygro cassette, using blunt-end ligation. This vector contains 8.5 kb of 5′ and 1.5 kb of 3′ homology (Fig. 1A).

Generation of *Otx2* mutant and wild-type ROSA26 ES cell lines

The culture, electroporation and selection of ES cells were carried out as described (Dierich and Dollé, 1997). The g3-hygro construct was electroporated after linearisation by *Sal*I into the ES cell line TE123 heterozygous for the $Otx2^{neo}$ mutation, whereby the homeodomain of *Otx2* was deleted and replaced by the PGKneo cassette (Ang et al., 1996). Electroporated cells were placed in a selective medium containing 150 μg/ml of hygromycin B. ES cell colonies that were resistant to hygromycin were analysed by Southern blotting to identify homologous recombination events at either the wild-type or the *neo* allele of the *Otx2* locus. Homologous recombination between the targeting vector g3-hygro and the wild-type or $Otx2^{neo}$ allele replaces the first and second exons of *Otx2*, including the ATG translation start site and most of the homeodomain or the PGKneo cassette, respectively, with the PGKhygro cassette from the pHA58 vector (te Riele et al., 1990), in a transcriptional orientation opposite to that of the *Otx2* gene, resulting in a new mutant allele of the *Otx2* gene called $Otx2^{hygro}$ (Fig. 1A). Genomic DNA from ES cells was digested with either *Eco*RI or *Nco*I and probed with a 4.0 kb *Xba*I-*Xba*I 3′ flanking fragment and with a 1.0 kb *Xho*I-*Xho*I 5′ flanking fragment, respectively (Fig. 1A). Of 144 colonies analyzed, 4 $Otx2^{neo/hygro}$ ($Otx2^{-/-}$) ES cell lines called TX3, TX62, TX81 and TX112 and 5 $Otx2^{hygro/+}$ ($Otx2^{+/-}$) ES cell lines called TX2, TX93, TX106, TX107 and TX111 were obtained, resulting from homologous recombination events occurring into the wild-type or $Otx2^{neo}$ allele, respectively (Fig. 1B and data not shown). The wild-type ROSA26/+ ES cell line, ES31 was isolated from blastocysts obtained from $Lim1^{-/+}$, ROSA26/+ intercrosses, as described (Robertson, 1987).

Generation and analysis of chimeras

The morulae-stage (E2.5) embryos used to generate chimeras were obtained from crosses of males homozygous for the ROSA 26 gene trap insertion (Friedrich and Soriano 1991) with CD1 females. Embryos were injected with approximately ten $Otx2^{-/-}$ (TX3, TX62, TX112), $Otx2^{+/-}$ (TX106 and TX111) or wild-type ES cells (R1 and H1) and reimplanted into pseudogestant females. The reciprocal type of chimeras were generated by injecting wild-type ROSA26/+ ES cells (ES31) into morulae isolated from $Otx2^{+/-}$ intercrosses. Chimeric embryos were harvested between E8.5 and E9.5, and processed for in situ hybridization or β-galactosidase staining as described in Beddington et al. (1989). After β-galactosidase staining, some embryos were processed for histological analysis as follows. Stained embryos were postfixed overnight in Bouin's fixative, rinsed in 70% ethanol, dehydrated and embedded in paraffin as described in Kaufman (1992). 7 μm thick sections were counterstained with 0.01% safranin-O that stained ES-derived cells in pink whereas embryo-derived cells were blue. In experiments using embryos from $Otx2^{+/-}$ intercrosses, the genotype of the host morula was determined retrospectively from VYS endoderm, that was isolated from the mesoderm layer following digestion in pancreatin and trypsin as described (Hogan et al., 1994). DNA samples prepared from the endodermal fraction were genotyped with respect to the *Otx2* locus by PCR as described (Ang et al., 1996).

Mouse strains

The $Otx2^{neo}$ allele was maintained on a CD1×129Sv/J background and animals genotyped as described (Ang et al., 1996). The $Lim1^{-}$ allele was maintained on a mixed (CD1×129Sv/J × C57BL/6) background.

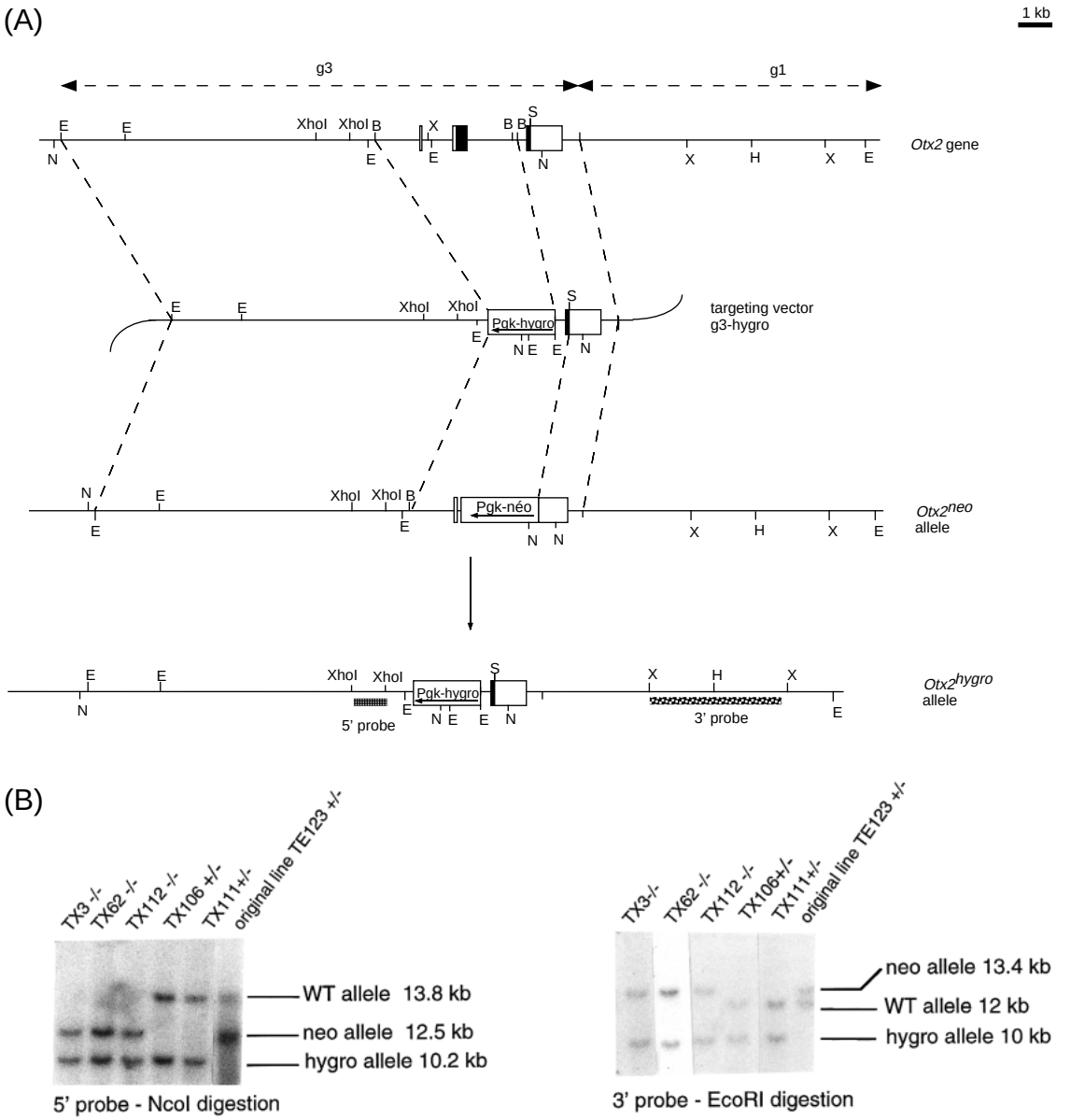

Fig. 1. Targeted disruption of the *Otx2* gene. (A) The open boxes represent the coding region and the solid boxes indicate the homeodomain of *Otx2*. The DNA fragments contained in bacteriophage clones g3 and g1 are shown as dotted arrows above the genomic structure of the *Otx2* gene. The 5′ and 3′ probes used for Southern blot analysis are indicated. If homologous recombination occurs on the wild-type allele, digestion of genomic DNA with *Eco*RI shifts the restriction fragment identified by the 3′ flanking probe from 12.0 kb to 10.0 kb and digestion with *Nco*I shifts the restriction fragment identified by the 5′ flanking probe from 13.8 kb to 10.2 kb. Alternatively, if homologous recombination occurs on the $Otx2^{neo}$ allele, digestion with *Eco*RI leads to a band shift from 13.4 kb to 10.0 kb using the 3′ flanking probe, while digestion with NcoI leads to a band shift from 12.5 kb to 10.2 kb using the 5′ flanking probe. B, *Bgl*II; E, *Eco*RI; N, *Nco*I; H, *Hin*dIII; S, *Stu*I; X, *Xba*I. (B) Southern blot analysis of DNA from *Otx2* mutant ES cells. The sizes of the DNA bands are indicated in kb. The 5′ and 3′ probes detected restriction fragments of predicted sizes for the wild-type, $Otx2^{hygro}$ and $Otx2^{neo}$ alleles. Abbreviations: WT, wild-type.

Mice carrying the ROSA26 gene trap insertion (Friedrich and Soriano, 1991) were obtained from the Jackson Laboratories, Bar Harbour.

Explant cultures

The ectoderm germ layer was separated from the mesendoderm of $Otx2^{-/-}$ and $Lim1^{-/-}$ embryos at E7.5 using tungsten needles, after incubating these embryos in 0.25% pancreatin, 0.5% trypsin in phosphate-buffered saline (PBS) at 4°C for 10 minutes. The whole ectoderm, except for the posterior part corresponding to the primitive streak, was used. Mesendoderm from the anterior half of wild-type embryos at E7.75 was isolated in a similar way and recombined with $Otx2^{-/-}$ or $Lim1^{-/-}$ ectoderm as described before (Ang and Rossant, 1993). Ectoderm from mutant embryos was also cultured alone in bacteriological dishes. All explants were cultured for 2 days, and fixed in 4% paraformaldehyde in PBS for 30 minutes and processed for whole-mount in situ hybridization or antibody staining.

In situ hybridization and immunohistochemistry

Embryos were collected and fixed in 4% paraformaldehyde in PBS for 1 hour, rinsed in PBS plus 0.1% Tween 20 and stored at –20°C in

methanol. Chimeric embryos were identified morphologically or by β-galactosidase staining of the entire embryo or the allantois. Whole-mount in situ hybridization was performed as described previously (Conlon and Herrmann, 1993). The following probes were used: *Six3* (Oliver et al., 1995), *Fkh2* (Kaestner et al., 1995), *Pax2* (Nornes et al., 1990), a 0.9 kb rat *chordin* cDNA (kindly provided by H. Wang), *follistatin* (Albano et al., 1994), a 1.3 kb *noggin* cDNA containing entire coding region (kindly provided by R. Harland and A. McMahon), *Wnt1* (Fung et al., 1985), *Sox1* (Collignon et al., 1996), *Hoxa2* and *Hoxb1* (Wilkinson et al., 1989) and *Mox1* (Candia et al., 1992). Whole-mount immunohistochemistry was performed as described in Davis et al. (1991). The En-specific antiserum (Davis et al., 1991) was used at a dilution of 1:1000. The secondary antibody was a peroxidase-coupled donkey anti-rabbit antibody (Jackson Immunoresearch) which was used at a dilution of 1:200.

RESULTS

Forebrain and midbrain are not induced in *Otx2*$^{-/-}$ embryos

We have previously reported that embryos homozygous for a mutation in *Otx2* consisting of a deletion of the homeobox region are missing the forebrain, midbrain and anterior hindbrain at early somite stages (Ang et al., 1996). To determine if this defect is due to a lack of formation of these tissues, which are normally induced by the underlying mesendoderm, or to a loss of the tissues shortly after their formation, we studied the expression of early markers of the anterior neural plate in *Otx2*$^{-/-}$ embryos. *Six3* is a homeobox gene that is first expressed only a few hours after formation of the anterior neural plate at E7.75 (headfold stage), in the presumptive forebrain region (Oliver et al., 1995). *Six3* was not expressed in *Otx2*$^{-/-}$ embryos of E7.5-8.25 (late-streak to early somite stages) (Fig. 2A,A′ and data not shown). *Fkh2*, a winged-helix gene expressed in the presumptive forebrain, midbrain and anterior axial mesoderm (Kaestner et al., 1995), was not expressed in the neural plate, but was observed in axial mesendoderm cells in the distal tip of *Otx2*$^{-/-}$ embryos at E7.75 (headfold stage) (Fig. 2B,B′). The lack of early markers of presumptive forebrain and midbrain strongly suggests a failure of induction of these tissues in *Otx2*$^{-/-}$ embryos rather than a loss of the tissues subsequently to their formation.

We also examined whether anterior hindbrain, and in particular presumptive rhombomere 1, was initially formed in *Otx2*$^{-/-}$ embryos, using *Pax2* (Rowitch and McMahon, 1995) as a marker of this region. *Pax2* was expressed in a broad domain corresponding to the midbrain and anterior hindbrain in wild-type embryos at E8.25 (Fig. 2C). *Pax2* was still expressed in *Otx2*$^{-/-}$ embryos, albeit in a reduced domain (Fig. 2C′). The reduced domain of *Pax2* expression is most likely due to expression in the anterior hindbrain of *Otx2*$^{-/-}$ embryos. Thus, in contrast to the lack of formation of forebrain and midbrain, anterior hindbrain tissue corresponding to rhombomere 1 appears to be formed initially but is subsequently deleted in *Otx2*$^{-/-}$ embryos at E8.5 (Ang et al., 1996).

noggin, chordin and follistatin are expressed in *Otx2*$^{-/-}$ embryos

The lack of formation of the anterior neural tissue in *Otx2*$^{-/-}$ embryos could result from a loss of neural-inducing molecules in the organizer, or node (Lemaire and Kodjabachian, 1996). We therefore studied the expression of three candidate neural-inducing genes, *noggin* (Smith and Harland, 1992), *chordin* (Sasai et al., 1994) and *follistatin* (Hemmati-Brivanlou et al., 1994), in mutant embryos. Although no organised node structure could be recognised, the three genes were expressed in the distal tip of *Otx2*$^{-/-}$ embryos, i.e. in the region corresponding to the node in wild-type embryos (Fig. 3A′-C′). *Noggin* and *chordin*, which are normally also expressed in the axial mesoderm at the anterior end of wild-type embryos (Fig. 3A,B), were missing in the anterior midline of mutant embryos (Fig. 3A′,B′). *Follistatin* was also expressed in the entire length of the primitive streak of both wild-type and mutant embryos (Fig. 3C,C′). These results confirm previous studies showing defects in the anterior axial mesoderm of *Otx2*$^{-/-}$ mutant embryos (Ang et al., 1996). They also demonstrate that the failure to induce forebrain and midbrain in *Otx2*$^{-/-}$ embryos is not due to a loss of expression of *noggin*, *chordin* or *follistatin* in the node.

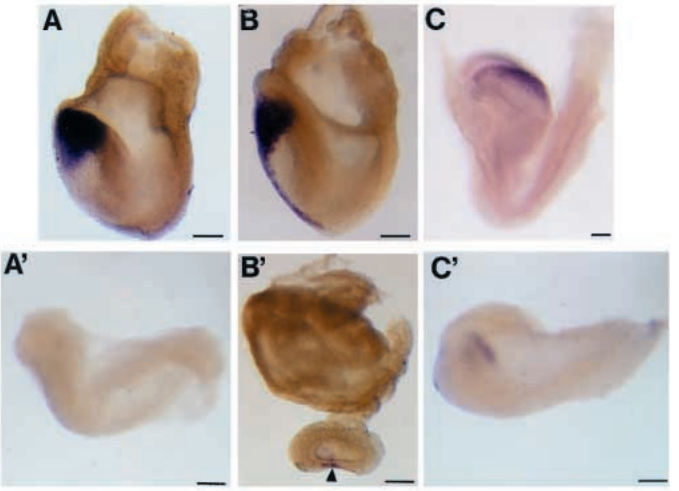

Fig. 2. Lack of expression of anterior neural plate markers in *Otx2*$^{-/-}$ embryos. Whole-mount RNA in situ hybridization of wild-type (A-C), and *Otx2*$^{-/-}$ (A′-C′) embryos. (A,A′) *Six3* expression at E7.75. *Six3* is expressed in the presumptive forebrain region in wild-type embryos (A) but is missing in mutant embryos (A′). (B,B′) *Fkh2* expression at E7.75. In wild-type embryos, *Fkh2* is expressed in the axial mesoderm and the presumptive forebrain and midbrain (B). In mutant embryos, *Fkh2* expression is restricted to axial mesoderm cells at the distal tip (arrowhead in B′). (C,C′) *Pax2* expression at E8.25. *Pax2* is expressed in the mid-hindbrain region in wild-type (C) and mutant (C′) embryos, but the expression domain in mutant embryos is smaller in size presumably due to the absence of the midbrain. Anterior is to the left. Scale bar: 100 μm.

Generation of strong *Otx2*$^{-/-}$↔*+/+* chimeras

Embryological studies in vertebrates have demonstrated that the formation and regionalization of the neural tube is dependent on interactions between the ectoderm and the underlying axial mesendoderm (Lemaire and Kodjabachian, 1996). In mouse, recent studies have also implicated the visceral endoderm in this process (Thomas and Beddington, 1996; Varlet et al., 1997). Since *Otx2* is expressed in all germ layers at the anterior end of the embryo during gastrulation (Simeone et al., 1993; Ang et al., 1994), the lack of anterior

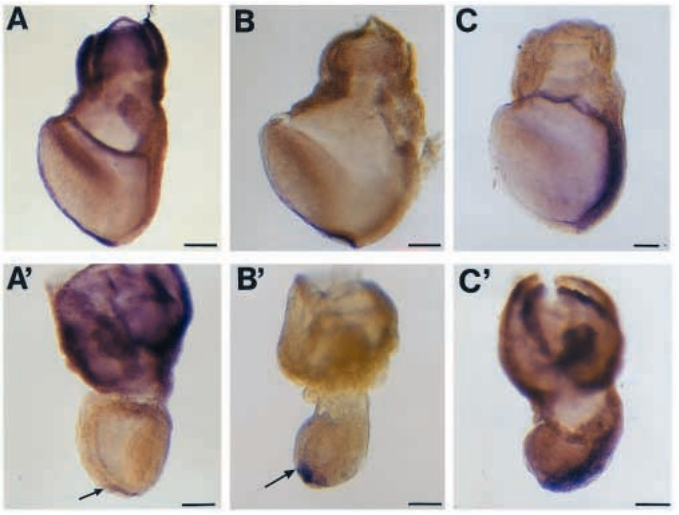

Fig. 3. Expression of signalling molecules in *Otx2*$^{-/-}$ embryos. Whole-mount RNA in situ hybridization of wild-type (A-C) and *Otx2* $^{-/-}$ embryos (A′-C′) at E7.75. (A,A′,B,B′) *noggin* (A) and *chordin* (B) are expressed in the axial mesoderm and node in normal embryos. In mutant embryos, *noggin* (A′) and *chordin* (B′) are expressed in the disorganised node but expression in the axial mesoderm does not extend as far anteriorly as in wild-type embryos. (arrows in A′,B′) *Follistatin* is expressed in the primitive streak of wild-type (C) and *Otx2*$^{-/-}$ mutant embryos (C′). Anterior is to the left. Scale bar: 100 μm.

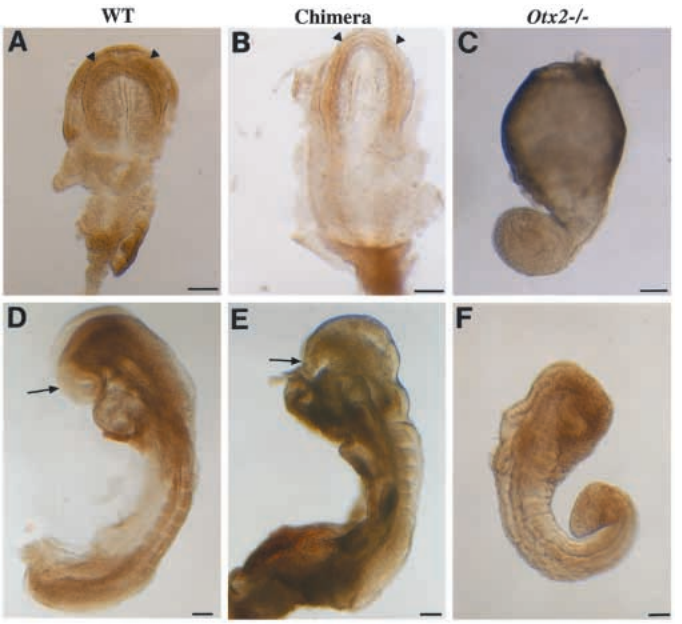

Fig. 4. Morphological analysis of *Otx2*$^{-/-}$↔+/+ chimeric embryos. (A,D) Wild-type, (B,E) *Otx2*$^{-/-}$↔+/+ and (C, F) *Otx2*$^{-/-}$ embryos. (A-C) 0- to 4-somite stage. *Otx2*$^{-/-}$↔+/+ chimeric embryos (B) show normal development of anterior neural folds (arrowheads), compared to wild-type embryos (A). This tissue is missing at the anterior end of *Otx2*$^{-/-}$ embryos (C). (D-F) 6- to 8-somite stage. *Otx2*$^{-/-}$↔+/+ chimeric embryos (E) show better development of the rostral brain than *Otx2*$^{-/-}$ embryos (F). However, the forebrain in chimeric embryos appeared less well developed than in wild-type embryos (arrows in E and D, respectively). Anterior is to the top, except for C where anterior is to the left. WT, wild-type. Scale bar: 100 μm.

neural plate in *Otx2*$^{-/-}$ embryos could be due to defects in the inducing anterior axial mesendoderm or visceral endoderm or in the responding ectoderm. To identify the tissues that require *Otx2* activity for anterior neural plate formation, we generated chimeric mouse embryos composed of *Otx2*$^{-/-}$ and wild-type cells, referred to as *Otx2*$^{-/-}$↔+/+ chimeras. These chimeras were produced by injecting three different *Otx2*$^{-/-}$ ES cell lines, called TX3, TX62 and TX112, into morula-stage embryos generated by crossing *Rosa26* homozygous males with CD1 females. The *Rosa26* transgenic mouse line carries a *lacZ* gene trap insertion that results in widespread *lacZ* expression during embryonic development (Friedrich et al., 1991). Thus, expression of *lacZ* was used to distinguish cells derived from wild-type embryos (*lacZ*-positive) from *Otx2*$^{-/-}$ ES-derived (*lacZ*-negative) cells. Reimplanted chimeric embryos were harvested between E8.5 and E9.5 and the level of chimerism was analysed by immunohistochemical staining for β-galactosidase activity. We have classified the chimeras as strong, moderate or weak depending on whether they contain more than 90%, 50% or 20%, respectively, of *Otx2*$^{-/-}$ cells. In this paper, we will report only our studies of the strong chimeras. In these chimeras, the embryo is almost entirely made up of *Otx2*$^{-/-}$ cells, while the VYS contains exclusively wild-type cells (see below). We were thus able to use these chimeras to address the function of *Otx2* in the visceral endoderm.

Rescue of anterior neural plate in strong chimeras

We harvested a total of 49 strong *Otx2*$^{-/-}$↔+/+ chimeras between E8.5 and E9.5. Strong chimeras (referred to below as chimeric embryos or chimeras) were developmentally delayed, and contained 0 to 14 somites (Figs 4B,E, 5C), compared with non-chimeric embryos or weak chimeras in the same litters (data not shown) and with control chimeric embryos generated by injection of +/+ or *Otx2*$^{+/-}$ ES cells (Fig. 5A,B), that had 12 to 40 somites. The delayed development of the chimeric embryos suggests that *Otx2* regulates the growth of the epiblast during gastrulation. Interestingly, all the chimeric embryos with less than 10 somites contained tissue anterior to rhombomere 2 (Fig. 4B,E), which was missing in *Otx2*$^{-/-}$ mutant embryos obtained from heterozygous intercrosses (Fig. 4C,F). In several chimeric embryos at the 0- to 4-somite stage, the rescued anterior neural plate appeared indistinguishable from that of wild-type embryos (compare panels B and A in Fig. 4). In contrast, the anterior neural plate in chimeric embryos with 6-8 somites appeared less well developed than in wild-type embryos at the same stage (Fig. 4D,E) and it could no longer be identified morphologically in chimeric embryos with 12-14 somites (data not shown). Thus, the development of the rostral neural plate appears to be initially rescued in chimeric embryos, but this tissue is progressively deleted at later stages.

Distribution of mutant and wild-type cells in strong chimeras

Ten chimeras containing 0-6 somites were analysed histologically in transverse sections. The neural tube of these

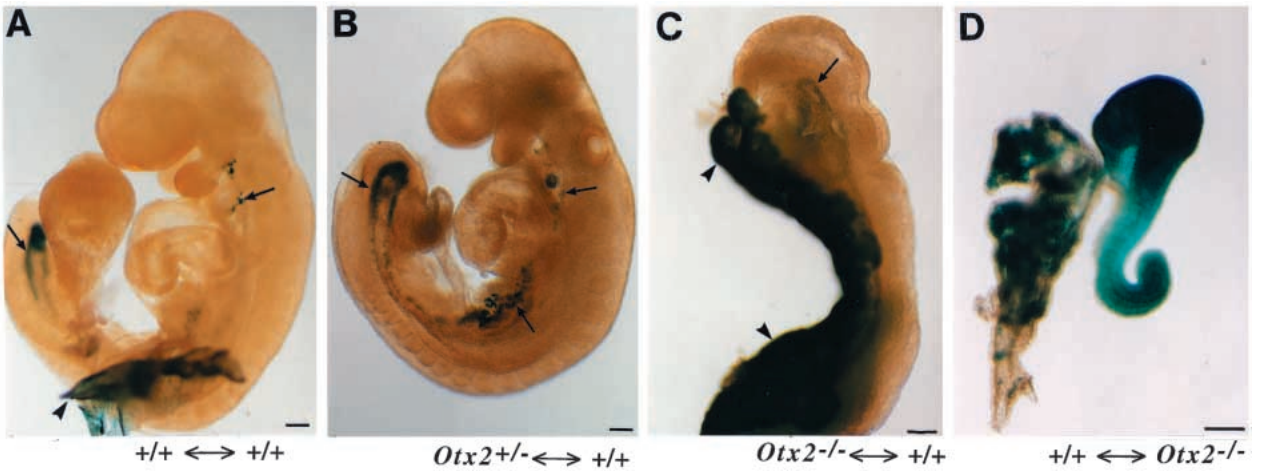

Fig. 5. Control chimeras. (A-C) Comparison of the distribution of embryonic cells and ES cells in strong chimeric embryos, generated by injection of +/+ (A), *Otx2*$^{+/-}$ (B) and *Otx2*$^{-/-}$ (C) ES cells into wild-type RosA26/+ embryos at E9.5 and stained for β-galactosidase activity. *lacZ*-positive, wild-type cells derived from the embryo are located exclusively in the gut (arrows) and VYS endoderm (arrowheads) in these different chimeras (A-C), irrespective of the genotype of the ES cells. (D) Reciprocal type of chimeras generated by injection of wild-type ROSA26/+ ES cells into a *Otx2*$^{-/-}$ morula at E8.5. *lacZ*-positive, wild-type ES cells have not colonized the VYS endoderm which is entirely *Otx2*$^{-/-}$ (see text), but have extensively colonized the embryo and mesoderm of the VYS. Nevertheless, the *Otx2* mutant phenotype is not rescued, thus demonstrating that this phenotype originates from a requirement for *Otx2* function in the visceral endoderm. Anterior is to the top. Scale bar: 100 μm.

chimeras was entirely composed of *Otx2*$^{-/-}$ cells (Figs 5C, 6D-I). The tissue rostral to the hindbrain had the characteristic morphology of a neuroepithelium, suggesting that it had a neural identity (Fig. 6D,G). *Otx2*$^{-/-}$ cells were also observed in the prechordal plate (Fig. 6D) and anterior notochord (Fig. 6E,H). Thus, *Otx2*$^{-/-}$ cells can contribute to all the tissues of strong chimeric embryos, including the rostral brain, prechordal plate and anterior notochord, which are missing in *Otx2*$^{-/-}$ embryos.

In chimeric embryos, wild-type cells were found exclusively in the endoderm of the VYS and of the gut along the entire A-P axis (blue cells, Fig. 6D-I). In two of the ten chimeras analysed, wild-type cells were in addition found in notochord cells (data not shown). In the gut endoderm, wild-type cells were intermingled with mutant cells and contributed from 10 to 50% of this germ layer (Fig. 6E-I). In contrast, the VYS endoderm contained exclusively wild-type cells. The absence of ES-derived cells in visceral endoderm derivatives was not surprising, since it has previously been shown that ES cells contribute very poorly to this tissue (Beddington and Robertson, 1989; Varlet et al., 1997). However, ES cells have not been shown to contribute less efficiently than embryonic cells to the gut endoderm.

We therefore examined whether the presence of wild-type cells in the gut endoderm of ES cell chimeras correlated with the mutation of *Otx2* in ES cells. Two +/+ ES cell lines, R1 (Nagy et al., 1993) and H1 (A. Dierich, unpublished), and two *Otx2*$^{+/-}$ ES cell lines, TX106 and TX111, were injected into morula-stage embryos. In chimeras generated with these +/+ and *Otx2*$^{+/-}$ ES cell lines, referred to as +/+↔+/+ and *Otx2*$^{+/-}$ ↔+/+, respectively, *lacZ*-positive embryo-derived cells were also found in the endoderm of the VYS and of the gut (Figs 5A,B, 6A-C), demonstrating that the presence of wild-type cells in the gut in chimeric embryos is not due to the *Otx2* mutation.

Wild-type visceral endoderm rescues anterior neural plate in chimeric embryos

Histological analyses of *Otx2*$^{-/-}$↔+/+ chimeras demonstrated that wild-type cells were always found in the VYS endoderm. In addition, wild-type cells were found exclusively in this tissue in 8/10 chimeras. These results suggest that *Otx2* is required in the visceral endoderm for the rescue of anterior neural plate development in chimeric embryos. To confirm this result, we generated the reciprocal type of chimeras, by injecting wild-type ROSA26/+ (*lacZ*-positive) ES cells into *Otx2*$^{-/-}$ embryos (referred to as +/+↔*Otx2*$^{-/-}$ chimeras). All 6 +/+↔*Otx2*$^{-/-}$ strong chimeric embryos examined at E8.5 exhibited the characteristic *Otx2* homozygous mutant phenotype (Fig. 5D), failing to develop a forebrain and midbrain. PCR analysis of the endoderm of the VYS of these chimeras confirmed the mutant genotype of the injected embryos and also showed that in four cases, the visceral endoderm contained exclusively *Otx2*$^{-/-}$ mutant cells (data not shown). Altogether, these results demonstrate that *Otx2* is required in the visceral endoderm for induction of forebrain and midbrain.

Lack of regional specification in the rescued anterior brain of chimeric embryos

To first confirm the neural nature of the rescued tissue rostral to the hindbrain of *Otx2*$^{-/-}$↔+/+ chimeras, we showed that it expressed *Sox1*, an early general neural marker (Collignon et al., 1996, Fig. 7A,A′). We next determined whether the rescued *Otx2*$^{-/-}$ neural tissue was properly regionalised. We analysed the expression of genes marking different anterior-posterior positions along the neural tube in chimeras between E8.5 and E9.5. *Six3*, an early forebrain marker (Oliver et al., 1995) was expressed in a reduced domain in chimeras containing 0-4 somites (Fig. 7B,B′). Its expression, however, was no longer detected in chimeras at the 6- to 8-somite stage whereas it was

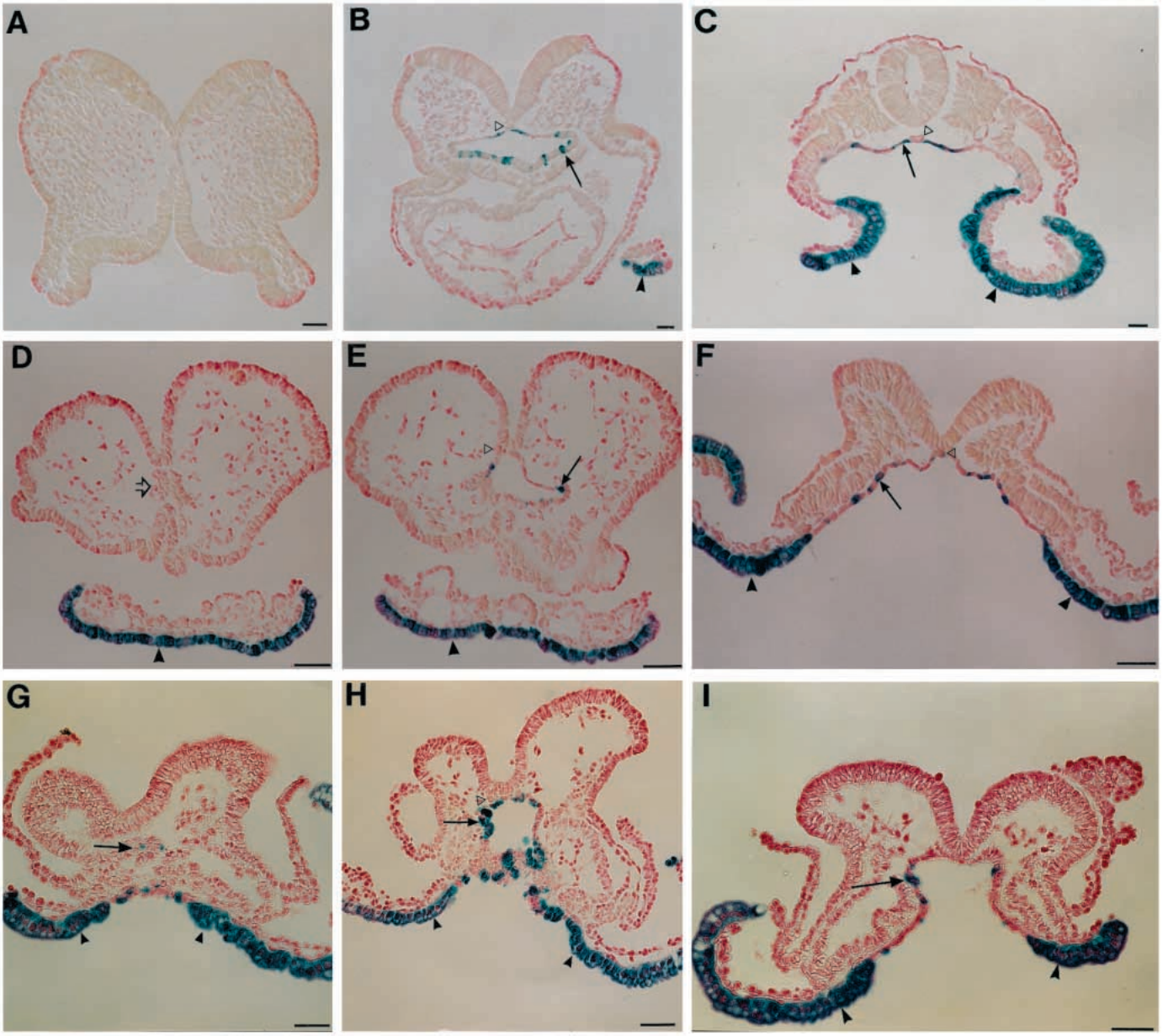

Fig. 6. Histological analysis of strong *Otx2*$^{-/-}$↔+/+ chimeric embryos. (A-C) +/+↔+/+ control chimera; (D-I). *Otx2*$^{-/-}$↔+/+ chimeras. (A,D,G) Transverse sections at the head level, (B,E,H) at the heart level and (C,F,I) at the spinal cord level. (D-I) The anterior neural tube in *Otx2*$^{-/-}$↔+/+ chimeras contains only *Otx2*$^{-/-}$ (pink) cells and presents a morphology characteristic of neuroepithelium. *Otx2*$^{-/-}$ cells also contribute to the prechordal plate (open arrow in Fig. 6D) and the anterior notochord (open arrowheads). In contrast, wild-type (blue) cells are localised exclusively in the endoderm of the VYS (arrowheads in B-I) and of the gut (arrows in B,C, E-I) in both +/+↔+/+ control chimeras (B,C) and *Otx2*$^{-/-}$↔+/+ chimeras (D-I). In the gut endoderm, the ES-derived cells are intermingled with wild-type cells, whereas the VYS is made up exclusively of wild-type cells (C-I). Scale bar: 100 μm.

normally expressed in the ventral diencephalon and optic vesicles of control embryos (7C,C′). In contrast, expression of *Hesx1/Rpx* (Thomas and Bedddington, 1996; Hermesz et al., 1996), another early forebrain-specific gene, was never observed in chimeric embryos, even between the early headfold and 4-somite stage (Fig. 7D,D′,E,E′ and data not shown). Therefore, different forebrain genes show different requirement for *Otx2* function. *Otx2* is required for the initiation of *Hesx1/Rpx* expression, and for the maintenance of *Six3* expression.

Expression of the regional markers for the mesencephalic-metencephalic (mes-met) region, *Pax2* (Nornes et al., 1990), *En* (Davis et al., 1991) and *Wnt1* (Rowitch et al., 1995), was also analysed in *Otx2*$^{-/-}$↔+/+ chimeric embryos. At the 0- to 4-somite stage, *Pax2* expression in the mes-met region was expanded rostrally compared to its expression in wild-type embryos (Fig. 8A,A′). At the 4-somite stage, En expression in chimeric embryos was weaker and more patchy than in wild-type embryos, and also appeared expanded rostrally (Fig. 8C,C′). By the 6- to 8-somite stage, *Pax2* and En

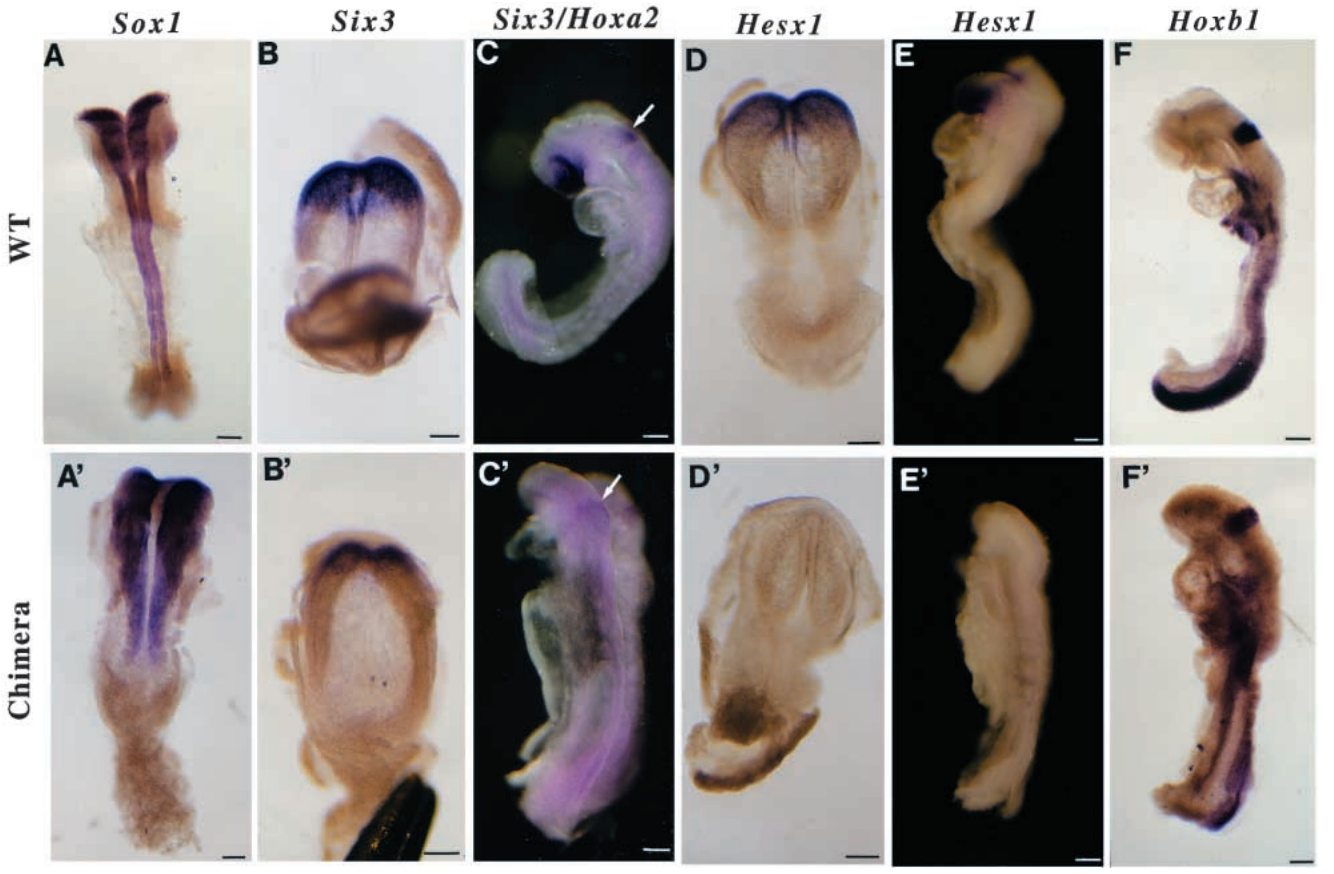

Fig. 7. Expression of the neural markers, *Sox1*, *Six3*, *Hoxa2*, *Hesx1/Rpx*, and *Hoxb1* in *Otx2*$^{-/-}$↔+/+ chimeras. Whole-mount RNA in situ hybridization analysis of wild-type embryos (A-F) and *Otx2*$^{-/-}$↔+/+chimeras (A′-F′). (A,A′) *Sox1* expression at the 6-8-somite stage. *Sox1* is similarly expressed in the neural tube of wild-type (A) and chimeric embryos (A′). (B,B′) *Six3* expression at the 0- to 4-somite stage. *Six3* is expressed in a reduced domain in the anterior neural plate (presumptive forebrain) of chimeric embryos (B′), compared to its expression in wild-type embryos (B). (C,C′) *Six3* and *Hoxa2* expression at the 6- to 8-somite stage. *Six3* is no longer detected in chimeric embryos (C), while it is still expressed in the forebrain and optic vesicles of wild-type embryos (C′). In contrast, *Hoxa2* is similarly expressed in rhombomere 3 (arrow) in wild-type (C) and chimeric (C′) embryos. (D,D′,E,E′) *Hesx1/Rpx* expression at the 0- to 4 (D) and 6- to 8 (E)-somite stages in the forebrain was not detected in chimeric embryos (D′,E′). (F,F′) *Hoxb1* is expressed in rhombomere 4 and the posterior spinal cord in both wild-type (F) and chimeric embryos at the 6- to 8-somite stage (F′). Anterior is to the top. WT, wild-type. Scale bar: 100 μm.

expression domains were not maintained except for a very small domain at the rostralmost tip of the chimeric embryos (Fig. 8B,B′,D,D′). *Wnt1* is normally expressed at E8.5 in a dorsal midline stripe along the neural tube and, in a transverse band, in the caudal mesencephalon. The dorsal expression domain of *Wnt1* was present, but its expression in the ventral mesencephalon was not detected in chimeric embryos containing 0 to 8 somites (Fig. 8E,E′,F,F′). Therefore, as for forebrain genes, the expression of mes-met genes is differentially affected by the loss of *Otx2* function. *Otx2* is required for the initiation of *Wnt1* expression in ventrocaudal mesencephalon and for the maintenance of *Pax2* and *En* expression in the mes-met region. In addition, En, which is normally expressed in wild-type embryos by the 1-somite stage (Davis et al., 1991), was not detected in chimeric embryos with 1-3 somites (data not shown), indicating a delay in the initiation of En expression in the absence of *Otx2*.

The expression of hindbrain-specific genes was also examined in chimeric embryos. Expression of the genes *Hoxa2* and *Hoxb1* (Wilkinson et al., 1989) in rhombomeres 3 (Fig. 7C,C′) and 4 (Fig. 7F,F′) respectively, were similar in wild-type and chimeric embryos at the 6- to 8-somite stage. At the 12-somite stage, expression of *Hoxa2* in rhombomere 3 was observed close to the rostral end of chimeras, indicating that tissues anterior to rhombomere 3 were deleted (data not shown). Therefore, expression of posterior neural markers occurred normally in the absence of *Otx2* expression.

Otx2 is required in the neurectoderm for Engrailed expression

Rescued anterior neurectoderm and axial mesoderm both contained exclusively mutant cells in most *Otx2*$^{-/-}$↔+/+ chimeric embryos. The defects in expression of forebrain and mes-met markers in these chimeras could therefore have two causes: it could be due to an autonomous requirement for *Otx2* in the neurectoderm, or to a requirement for *Otx2* in the axial mesoderm for the induction or maintenance of expression of regional markers in the brain. To distinguish between these two hypotheses and specifically to study the function of *Otx2* in the neurectoderm, we performed culture experiments involving the recombination of ectoderm from

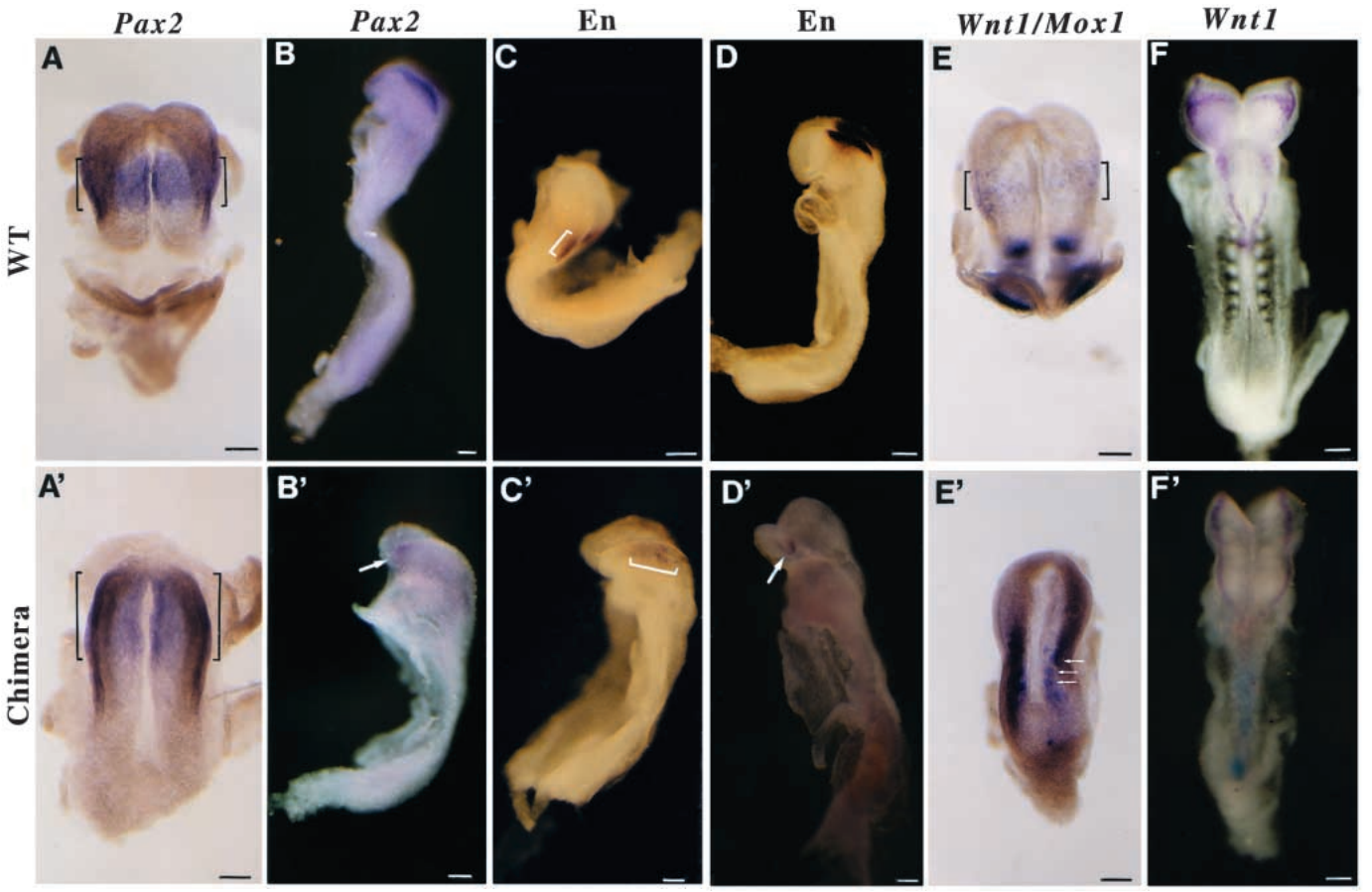

Fig. 8. Expression of mes-met markers in *Otx2*$^{-/-}$↔+/+ chimeras. Labelling of wild-type embryos (A-F) and *Otx2*$^{-/-}$↔+/+ chimeras (A′-F′). (A,A′) *Pax2* expression at the 0- to 4-somite stage. *Pax2* RNA expression in the presumptive mes-met region (brackets) is enlarged towards the rostral end in chimeric embryos (A′), compared to its expression in wild-type embryos. (B,B′) *Pax2* RNA expression at the 6- to 8-somite stage. *Pax2* is expressed in a small domain at the rostral end (arrow) of chimeric embryos, whereas it is expressed in two separate domains in the forebrain and mes-met region of wild-type embryos. (C,C′) En protein expression at the 4-somite stage. En is expressed in the mes-met region of wild-type embryos (bracket in C), and has a weaker and more patchy expression domain, which is expanded rostrally in chimeric embryos (bracket in C′). (D,D′) En protein expression at the 6- to 8-somite stage. En is expressed in the mes-met of wild-type embryos (D), but only at the rostralmost tip of the neural tube of chimeric embryos (arrow in D′). (E,E′) *Wnt1* and *Mox1* expression at the 0- to 4-somite stage. *Wnt1* is expressed in a weak and broad domain in the mesencephalon of wild-type embryos (brackets in E), but not in a chimeric embryo with three somites expressing *Mox1* (small arrows in E′). (F,F′). *Wnt1* expression at the 6- to 8-somite stage. *Wnt1* is expressed in the dorsal neural tube but not in a transverse band in the posterior mesencephalon of chimeric embryos (F′), whereas it is expressed in both these regions in wild-type embryos (F). Anterior is to the top. WT, wild-type. Scale bar: 100 μm.

Otx2$^{-/-}$ embryos with axial mesendoderm from wild-type embryos. We have previously shown in similar explant recombination experiments that anterior mesendoderm from wild-type embryos can induce expression of the mes-met marker En in posterior lateral ectoderm isolated from wild-type embryos (Ang and Rossant, 1993). Thus, posterior ectoderm cells, whose normal fate is spinal cord and surface ectoderm, can be instructed to adopt a mes-met fate by anterior mesendoderm. Using this assay, we asked whether *Otx2* is required in the neuroectoderm for the expression of En. The ectoderm germ layer from *Otx2*$^{-/-}$ embryos at E7.5 was recombined with the anterior mesendoderm from E7.75 wild-type embryos. These explants were cultured for 2 days and then assayed for expression of En proteins by whole-mount antibody staining.

All ten explants of *Otx2*$^{-/-}$ ectoderm recombined with anterior mesendoderm from wild-type embryos failed to express En after culture (Fig. 9B). Six control explants of *Otx2*$^{-/-}$ ectoderm cultured alone for 2 days also did not express En (Fig. 9A). Lack of En expression was not due to a failure of *Otx2*$^{-/-}$ ectoderm cells to adopt a neural fate, since *Otx2*$^{-/-}$ ectoderm isolated from E7.5 embryos and cultured for 2 days expressed the general neural marker, *Sox1* (Fig. 9C). As a control experiment for the efficiency of germ layer interactions in this culture system, we recombined ectoderm from *Lim1*$^{-/-}$ embryos with wild-type anterior mesendoderm. *Lim1* is a LIM and homeobox domain-containing gene that is expressed in the axial mesendoderm but not in the ectoderm or the neural plate (Barnes et al., 1994). *Lim1*$^{-/-}$ embryos present a loss of brain tissue rostral to rhombomere 3, similar to that of *Otx2*$^{-/-}$ embryos (Shawlot and Behringer, 1995). When we recombined *Lim1*$^{-/-}$ ectoderm with wild-type mesendoderm, En expression was rescued in 3 out of 6 mutant ectoderm explants (Fig. 9D). These results

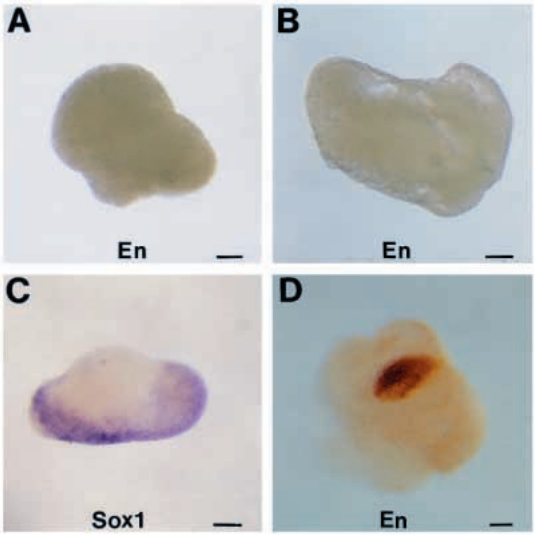

Fig. 9. Expression of En and *Sox1* in explants after culture for 48 hours. (A) Ectoderm explants from E7.5 *Otx2*$^{-/-}$ embryos fail to express En. (B) Ectoderm explants from E7.5 *Otx2*$^{-/-}$ embryos recombined with anterior mesendoderm from E7.75 wild-type embryos do not express En. (C) Ectoderm explants from E7.5 *Otx2*$^{-/-}$ embryos showing expression of the general neural marker, *Sox1*. (D) Ectoderm explants from E7.5 *Lim1*$^{-/-}$ embryos, recombined with anterior mesendoderm from E7.75 wild-type embryos, express En. Scale bar: 100 μm.

demonstrate that *Lim1* is not required in the neurectoderm for En expression, and that the loss of the mes-met region in *Lim1*$^{-/-}$ embryos must be due to a requirement for *Lim1* in axial mesendoderm or visceral endoderm, two tissues that normally expresses *Lim1* during gastrulation (Barnes et al., 1994; our unpublished results). In contrast, *Otx2* function is required in the neurectoderm for expression of En proteins. Specifically, since the explants were analysed for En expression 2 days after culture at a stage corresponding to approximately E9.0-E9.5, the recombination assay measures the requirement for *Otx2* in the maintenance rather than the initiation of En expression, which occurs in mouse embryos at E8.0. Thus, *Otx2* is required autonomously in the neurectoderm for the maintenance of En expression, in agreement with the results obtained from chimeric studies.

DISCUSSION

In this paper, we show that the absence of rostral brain in *Otx2*$^{-/-}$ embryos is due to a failure in the induction of the anterior neural plate at early stages of gastrulation. In strong *Otx2*$^{-/-}$↔+/+ chimeras, induction of forebrain and midbrain is rescued by the presence of wild-type cells in the visceral endoderm. Altogether, these results demonstrate that *Otx2* is required in the visceral endoderm for induction of the rostral brain. Using chimera studies and an explant recombination assay, we then demonstrate that *Otx2*$^{-/-}$ ectodermal cells can acquire some but not all the characteristics of anterior neuroectodermal cells. Therefore, *Otx2* has a second function in the neuroectoderm for the specification of the forebrain and mes-met regions of the neural tube.

Otx2 is required in the visceral endoderm for induction of forebrain and midbrain

In previous studies, we and others demonstrated that *Otx2*$^{-/-}$ embryos lack neural tissue rostral to rhombomere 3 at E8.5. Using early markers of the anterior neural plate, we now show that the presumptive forebrain and midbrain do not form while a presumptive rhombomere 1 expressing *Pax2* appears to be generated in *Otx2*$^{-/-}$ embryos at E7.75. Thus, the loss of neural tissue at this early stage correlates with the domain of *Otx2* expression in the neural tube, which ends precisely at the mid-hindbrain boundary (Millet et al., 1996). The absence of expression of *Six3* and *Fkh2* in *Otx2*$^{-/-}$ embryos indicates that the defect in anterior neurectoderm occurs very early, presumably during the phase of induction of this tissue through ectoderm-mesendoderm interactions.

Our chimeric studies provide strong evidence for a role of *Otx2* in the induction of forebrain and midbrain tissues. In chimeras, a general inability of ES cells to contribute to visceral endoderm leads to the preferential localisation of embryo-derived wild-type cells in this tissue along the whole anterior-posterior axis of the embryos. In strong *Otx2*$^{-/-}$↔+/+ chimeras (made of more than 90% *Otx2*$^{-/-}$ cells), the neural tissue rostral to the hindbrain always developed and was composed entirely of *Otx2*$^{-/-}$ cells, whereas wild-type cells were found exclusively in the visceral and gut endoderm in 8 out of 10 chimeras. Furthermore, the reciprocal chimeras, obtained by injecting +/+ cells into *Otx2*$^{-/-}$ embryos and having a visceral endoderm containing only *Otx2*$^{-/-}$ mutant cells, showed no rescue of rostral brain development. These results demonstrate that *Otx2* function is required in the visceral endoderm for development of forebrain and midbrain. Whether wild-type cells in the gut are also involved in the rescue remains to be determined, since it is not possible to generate chimeric embryos whose gut endoderm is entirely made up of *Otx2*$^{-/-}$ cells to test this hypothesis.

Our experiments provide compelling evidence for a role of *Otx2* in an interaction between visceral endoderm and ectoderm, necessary for the induction of forebrain and midbrain in mouse (Thomas and Beddington, 1996; Varlet et al., 1997). The nature of the signalling molecules regulated by *Otx2* and involved in this interaction remains to be determined. Two candidates are *nodal*, a TGFβ-related growth factor involved in anterior neural plate patterning (Varlet et al., 1997), and the secreted molecule *cerberus*, which is expressed in anterior endoderm and has the property to induce ectopic head structures when microinjected into ventral regions of *Xenopus* embryos (Bouwmeester et al., 1996). A mouse *cerberus* homologue (*mCer-1*) has recently been isolated and present an abnormal and reduced expression domain in the visceral endoderm of *Otx2*$^{-/-}$ embryos (Biben et al., 1997).

Otx2 is required in the neuroectoderm for its regional specification

To study *Otx2* function in the neuroectoderm, we have further characterised the anterior neural tissue that is rescued in strong chimeric embryos. This tissue, entirely made of *Otx2*$^{-/-}$ cells, expresses *Sox-1* and acquires some measure of regional patterning. Expression of *Six3* is initiated, albeit in a reduced domain, but is not maintained. Expression of *Hesx1/Rpx* is not initiated in the forebrain. These results indicate that a forebrain tissue is formed in absence of *Otx2*, but that *Otx2* is required

for its regional specification as early as the 0- to 4-somite stage. In addition, the expression domains of mes-met markers, such as *Pax2* and En, are expanded rostrally in chimeric embryos at this stage. This result, together with the reduced domain of *Six3* expression, suggest that part of the presumptive forebrain has adopted a more posterior fate and that *Otx2* may be required to prevent forebrain cells adopting a mes-met fate. By the 6- to 8-somite stage, a neural tissue expressing forebrain markers is missing altogether, indicating that the forebrain has either been eliminated or completely transformed to a more posterior fate. Thus, *Otx2* is also required after the initial stage of induction and specification, for maintaining regional identity in the forebrain and/or maintaining the tissue itself.

Pax2 and En are initially expressed in the mes-met region of *Otx2* chimeras, suggesting that establishment of this territory occurs in the absence of *Otx2* function. However, *Otx2* is required within this territory for the distinction between mesencephalic and metencephalic fates, since in its absence, expression of *Wnt1*, a gene required for development of the mesencephalon (McMahon et al., 1992), is not initiated. In addition, *Pax2* and En expression are almost completely lost by the 6- to 8-somite stage, indicating that *Otx2* is also required for the maintenance of the mes-met region. Loss of En expression could in part be caused by the loss of *Wnt1* expression, since *Wnt1* is required for the maintenance of En expression (McMahon et al., 1992; Danielian and McMahon, 1996). Interestingly, the regulation of En and *Wnt1* by *Otx2* appears to be evolutionarily conserved since *en* and *wg* are targets of the *Otx* homolog, *Otd*, in flies (Cohen and Jurgens, 1991).

Analysis of regional markers demonstrate important roles for *Otx2* in establishing regional identities in both forebrain and midbrain. One mechanism by which *Otx2* could establish distinct fates in these two regions could be by regulating gene expression in combination with other regionally expressed genes. In the forebrain, *Otx2* would interact with a coactivator to initiate expression of *Hesx1/Rpx*. In the mes-met region, *Otx2* would interact with another coactivator, possibly *Pax2*, to initiate *Wnt1* expression and consequently specify mesencephalic fates. This hypothesis raises the possibility that *Hesx1/Rpx* and *Wnt1* are direct targets of *Otx2*. By studying *Hesx1/Rpx* and*Wnt1* expression in moderate $Otx2^{-/-}\leftrightarrow+/+$ chimeras, in which the neuroectoderm contains a mosaic of mutant and wild-type cells, we should be able to determine if *Otx2* is required cell-autonomously or non-autonomously for the expression of these genes.

In our discussion so far, we have assumed that *Otx2* functions in the neuroectoderm for establishing forebrain and midbrain identity. However, we cannot exclude the possibility that some of the neural plate patterning defects of the chimeras are due to a requirement for *Otx2* in the prechordal plate. We have nevertheless addressed the specific requirement for *Otx2* function in the neuroectoderm for the expression of one regional marker, using explant-recombination experiments. These experiments demonstrate that *Otx2* is required in the neurectoderm for the maintenance of En expression.

In strong $Otx2^{-/-}\leftrightarrow+/+$ chimeras, the prechordal plate and anterior notochord contained exclusively $Otx2^{-/-}$ cells and nevertheless developed normally (expression of *HNF-3β* in these tissues was normal, data not shown). Therefore, *Otx2* is not required cell-autonomously for the formation of these tissues. In addition, *Otx2* is also not required in axial mesoderm and/or node for expression of signalling molecules such as chordin, noggin and follistatin. Our experiments, however, cannot rule out that *Otx2* has also a function in the prechordal plate for regionalization of the neural tube, since *Otx2* activity was missing in both the neuroepithelium and axial mesoderm of strong $Otx2^{-/-}\leftrightarrow+/+$ chimeras. Alternative approaches, involving the specific knock-out of *Otx2* function in different germ layers, will be necessary to study if *Otx2* also has a function in the prechordal plate.

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