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Cell autonomous and non-cell autonomous functions of *Otx2* in patterning the rostral brain

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SUMMARY

Previous studies have shown that the homeobox gene *Otx2* is required first in the visceral endoderm for induction of forebrain and midbrain, and subsequently in the neurectoderm for its regional specification. Here, we demonstrate that *Otx2* functions both cell autonomously and non-cell autonomously in neurectoderm cells of the forebrain and midbrain to regulate expression of region-specific homeobox and cell adhesion genes. Using chimeras containing both *Otx2* mutant and wild-type cells in the brain, we observe a reduction or loss of expression of *Rpx/Hesx1*, *Wnt1*, *R-cadherin* and *ephrin-A2* in mutant cells, whereas expression of *En2* and *Six3* is rescued by surrounding wild-type cells. Forebrain *Otx2* mutant cells subsequently undergo apoptosis. Altogether, this study demonstrates that *Otx2* is an important regulator of brain patterning and morphogenesis, through its regulation of candidate target genes such as *Rpx/Hesx1*, *Wnt1*, *R-cadherin* and *ephrin-A2*.

Key words: Forebrain, Midbrain, Patterning, Chimeras, Mouse, Adhesion molecules, R-cadherin, Ephrins

INTRODUCTION

The genetic and cellular mechanisms that control the formation and subdivision of the anterior neural plate into its major units, prosencephalon, mesencephalon and metencephalon, remain to be elucidated. Embryological and gene manipulation studies have shown that three groups of proteins, transcription factors, signalling molecules and cell surface molecules, are implicated in these processes (reviewed by Lumsden and Krumlauf, 1996). In particular, the identification of murine homologs of *Drosophila* homeobox genes that are expressed in the anterior segments of flies, such as *orthodenticle* (*otd*) and *empty spiracles* (*ems*), have greatly improved our understanding of this field (reviewed by Cohen and Jurgens, 1991a; Finkelstein and Perrimon, 1991, Finkelstein and Boncinelli, 1994). Murine homologs of *otd* and *ems*, called *Otx1* and *Otx2*, and *Emx1* and *Emx2* respectively, have been shown to be expressed in nested domains in the embryonic neural tube (Simeone et al., 1992). A third *orthodenticle*-related gene *Crx* has recently been isolated in the mouse and is expressed specifically in photoreceptor cells (Furukawa et al., 1997; Swain et al., 1997).

Mutations of the *Otx* and *Emx* genes in mice have demonstrated their essential roles in brain development, with *Otx2* null embryos the most severely affected, demonstrating that *Otx2* is required at an earlier stage than the other genes, i.e. during gastrulation (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996; Acampora et al., 1996; Pellegrini et al., 1996; Yoshida et al., 1997). Most importantly, the forebrain, midbrain and anterior hindbrain fail to develop in *Otx2* mutant embryos. In a previous study, based on the generation of mouse chimeras, we have demonstrated that *Otx2* is required in the visceral endoderm and not in the ectoderm for the establishment of the forebrain and midbrain territories (Rhinn et al., 1998). When *Otx2* activity is given back to the visceral endoderm in *Otx2–/–↔+/+* chimeric embryos generated by injection of *Otx2–/–* ES cells into wild-type (WT) morula-stage mouse embryos, chimeric embryos develop an anterior neural plate which is made of *Otx2* mutant cells but expresses early forebrain and midbrain characteristics. The *Otx2* mutant anterior neural plate is not maintained, however, but is deleted and/or transformed into a more posterior fate. Loss of brain regions in *Otx2* mutant embryos indicates that *Otx2* functions during gastrulation as a gap gene, in a manner analogous to its *Drosophila* homolog *otd* (Finkelstein and Perrimon, 1991; Cohen and Jurgens, 1991b), for the specification of forebrain and midbrain.

Otx2 is expressed in the neural plate after gastrulation, similar to the expression of *otd* in the brain anlage, and this neural expression has led to the hypothesis that *otd/Otx2* could also function later in the brain to specify regional identity. We have previously shown using *Otx–/–↔+/+* chimeric mice that *Otx2* is required in neurectodermal cells for expression of neural region-specific markers such as the homeobox gene *Rpx* and the signalling molecule *Wnt1* (Rhinn et al., 1998). These studies however did not allow us to determine whether *Otx2* function was required cell autonomously or non-cell autonomously.

It has been recently proposed that homeobox genes also

participate in the establishment of distinct territories in the brain through the regulation of cell adhesion molecules (Edelman and Jones, 1998; Stoykova et al., 1997). In the mesencephalic-metencephalic (mes-met) region these two territories are initially specified as one segment, which is subsequently subdivided through the activity of patterning signals that are produced at the boundary between the mesencephalon and the metencephalon, known as the isthmic organizer (reviewed by Bally-Cuif and Wassef 1995; Joyner, 1996). A role for *Otx2* in subdividing the mes-met region of the brain into the mesencephalic and metencephalic territories and in specifying the mesencephalic fate has been suggested by transplantation experiments in chick showing that *Otx2* marks the boundary between the mesencephalon and metencephalon from a very early stage (Millet et al., 1996). More direct evidence for a role of *Otx2* in specifying the mesencephalon has been provided by analyses of the phenotype of double *Otx1/Otx2* mutants (Acampora et al., 1997; Suda et al., 1997) and of chimeric *Otx2–/–↔+/+* mouse embryos (Rhinn et al., 1998). Early specification of regional identity in the mes-met territory presumably requires a mechanism to ensure that cells in the two adjacent territories do not mix. One potential mechanism to partition the mesencephalic and metencephalic units would be that *Otx2* regulates cell surface properties in the mesencephalon, resulting in differential affinities between mesencephalic and metencephalic cells.

We have used chimeric embryos containing both *Otx2* mutant and WT cells to distinguish cell autonomous versus non-cell autonomous functions of *Otx2* in regulating expression of region-specific genes in the forebrain and midbrain, and to examine changes in cell surface properties of *Otx2* mutant cells. Our results demonstrate that *Otx2* functions in the anterior brain and regulates expression of *Rpx* and *Wnt1* in a cell autonomous manner, suggesting that these genes could represent direct transcriptional targets of *Otx2*. Our work has also revealed a role for *Otx2* in regulating cell surface properties, through regulation of the cell adhesion molecule *R-cadherin* and a member of the ephrin family of ligands, *ephrin-A2*. By this mechanism, *Otx2* could establish a sharp boundary between mesencephalic and metencephalic cells. Finally, *Otx2* appears to be also required for the survival and maintenance of forebrain cells.

MATERIALS AND METHODS

Generation of *Otx2* mutant and WT ROSA26 ES cell lines

The culture, electroporation and selection of ES cells were carried out as described by Dierich and Dollé (1997). The generation of $Otx2^{neo/hygro}$ (Otx2–/–) ES cell lines (TX3, TX62 and TX112) have been described previously (Rhinn et al., 1998).

Generation and analysis of chimeras

The morulae-stage (E2.5) embryos used to generate chimeras were obtained from crosses of males homozygous for the ROSA26 gene trap insertion (Friedrich and Soriano, 1991) with CD1 females. Embryos were injected with approximately five to ten *Otx2–/–* (TX3, TX62, TX112) or WT R1 ES cells (Nagy et al., 1993) and reimplanted into pseudogestant females to generate *Otx2–/–↔+/+* and control chimeric embryos respectively. Chimeric embryos were harvested between embryonic day 8.25 (E8.25) and E9.5, and processed for in situ hybridization or β-galactosidase staining as described by Beddington et al. (1989). After β-galactosidase staining, TUNEL labelling and/or whole-mount in situ hybridization, some embryos were processed for histological analysis as follows. Stained embryos were post-fixed overnight in Bouin's fixative, rinsed in 70% ethanol, dehydrated and embedded in paraffin wax as described by Kaufman (1992). 7 μm thick sections were counterstained with 0.01% safranin-O that stained ES-derived cells pink whereas embryo-derived cells were blue. Double-labelling of chimeric embryos involving histochemical staining for β-galactosidsase activity followed by non-radioactive in situ hybridization with digoxigenin probes was performed as described by Houzelstein and Tajbakhsh (1998).

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: N-cadherin (Miyatani et al., 1989); a 3.3 kb R-cadherin cDNA (kindly provided by Dr Masatoshi Takeichi); Rpx/Hesx1 (Thomas and Beddington, 1996); Six3 (Oliver et al., 1995); Wnt1 (Fung et al., 1985); En2 (Davis and Joyner, 1988) and ephrin-A2 (Cheng and Flanagan, 1994; Gale et al., 1996).

TUNEL and BrdU experiments

The TUNEL procedure on whole embryos was performed after β-galactosidase staining as described by Conlon et al. (1995). For BrdU incorporation experiments, pregnant females were injected intraperitoneally with 100 μg BrdU/g body weight and killed after 1 hour. Embryos were processed and BrdU labelling was revealed as described in Mishina et al. (1995).

RESULTS

Maintenance of forebrain and midbrain development in chimeric embryos containing both *Otx2* mutant and WT cells

As previously reported, we have isolated several *Otx2–/–* embryonic stem (ES) cell lines, and used them to produce *Otx2–/–↔+/+* chimeric embryos by injection into ROSA26 *lacZ+/–* morulae. *lacZ* expression in ROSA26 transgenic mice is ubiquitous throughout early to mid-gestational development (Friedrich and Soriano, 1991) and is therefore a marker for WT cells in chimeric embryos. We used β-galactosidase activity to examine the contribution of WT and mutant cells in the *Otx2–/–↔+/+* chimeric embryos. We have shown that in chimeras containing only mutant cells in the embryo (referred to as strong chimeras), development of the mutant forebrain and midbrain is rescued until the 0- to 4-somite stage by the presence of WT cells in the visceral endoderm (Rhinn et al., 1998). However, by E8.5 (6- to 8-somite stage), the mutant rostral brain is progressively deleted or acquires a more posterior fate, and the rostral end of these embryos ectopically expresses markers of the isthmic region such as the paired box gene *Pax2*. Thus, the chimeras containing a high proportion of *Otx2* mutant cells cannot be used to examine the function of *Otx2* in forebrain and midbrain development after E8.5.

To study the functions of *Otx2* in the rostral brain beyond this stage, we have generated chimeras containing 25-50% of *Otx2* mutant ES cells (referred to hereafter as moderate

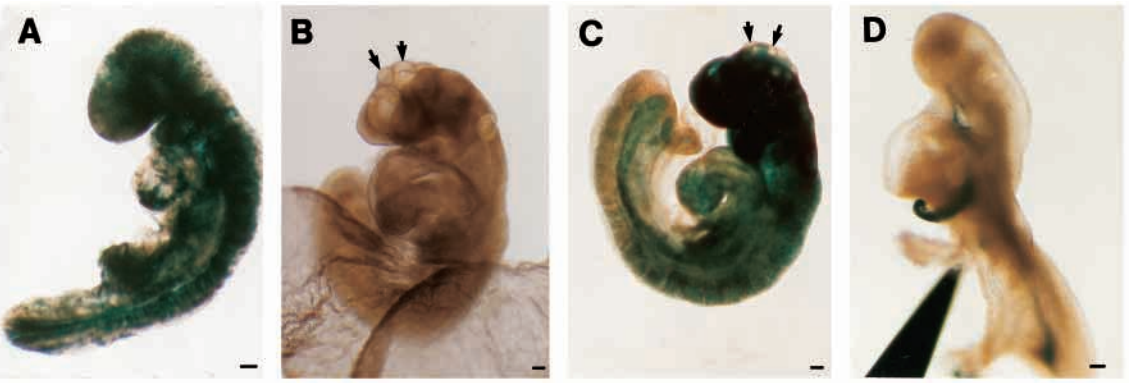

Fig. 1. Morphology of moderate *Otx2–/–↔+/+* chimeric embryos compared with strong *Otx2–/–↔+/+* and control chimeras at E9.5. (A) Control, (B,C) moderate *Otx2–/–↔+/+* and (D) strong *Otx2–/–↔+/+* chimeras. Embryos in A, C and D have been stained for β-galactosidase activity. (A) A control chimera showing normal development and a fine mosaic of ES-derived (white) and embryo-derived (blue) cells. (B-D) Forebrain and midbrain tissues are present in moderate chimeras (B,C). In contrast, these regions of the neural tube cannot be identified morphologically in the strong chimeric embryo shown in D. Although moderate chimeras present a better rescue of forebrain and midbrain development than strong chimeras, abnormal bulges are apparent in their rostral brain (arrows in B) and *Otx2* mutant cells (white) form patches (arrows in C) that are segregated from WT cells (blue). Scale bars, 100 μm.

chimeras). At E9.5, the major subdivisions of the brain, prosencephalon, mesencephalon and metencephalon, are easily identifiable in moderate chimeras (Fig. 1B,C), whereas strong chimeras lack these brain regions at the same stage (Fig. 1D). β-galactosidase staining of chimeric embryos shows that WT cells contribute to the embryo proper in moderate and not in strong chimeras. For example, the neural tube of moderate chimeras is highly mosaic, containing both *lacZ*-positive WT and *lacZ*-negative *Otx2* mutant cells (Fig. 1C). Thus, the improved rescue of forebrain and midbrain development in moderate chimeras compared with the strong ones is due to the presence of WT cells in embryonic tissues, most likely in the anterior neural tube and/or axial mesendoderm.

Moderate chimeras are not morphologically normal, however. When compared to control chimeras resulting from injection of WT ES cells into ROSA26 morulae (Fig. 1A), their anterior brain appears reduced in size and has a pointed appearance (Fig. 1B,C). In addition, the neuroepithelium presents abnormal bulges and the neural tube often fails to close (Fig. 1B). The severity of these phenotypes is correlated with the number of mutant cells contributing to the chimeras (data not shown). The maintenance of forebrain and midbrain territories in moderate chimeras at E9.5 has allowed us to examine the functions of *Otx2* in the brain at later stages than in previous studies, and to distinguish between cell autonomous and non-cell autonomous functions of *Otx2* in the anterior neuroepithelium.

Otx2 mutant cells fail to contribute to the forebrain, but not to the midbrain and hindbrain

Analysis of *lacZ* expression in moderate chimeras at E9.5 revealed striking patterns of mutant cell distribution in the brain. Sections through the brain showed that mutant cells are either completely absent from the forebrain (*n*=4) (Fig. 2E') or only present in very few patches (*n*=3) (data not shown). In contrast, mutant cells are largely present in the midbrain, and hindbrain of the same chimeras (Fig. 2D'-F'). Thus, *Otx2* mutant cells are specifically excluded from the forebrain of chimeric embryos at E9.5.

We also analysed the distribution of mutant cells in moderate chimeras at E8.5, to determine whether mutant cells initially contribute to the forebrain of moderate chimeras. Transverse sections showed that a few patches of mutant cells are always present in the forebrain at this stage (*n*=9), although it is already apparent that the contribution of mutant cells is higher in more posterior brain regions (Fig. 2A'-C'). This result is in agreement with our previous study demonstrating that *Otx2* mutant cells are initially able to contribute to the forebrain of strong chimeras (Rhinn et al., 1998). The loss of mutant forebrain cells between E8.5 and E9.5 could be either due to their death by apoptosis and/or to lower rates of proliferation compared with WT cells.

BrdU incorporation experiments were performed to determine if *Otx2* mutant cells have a reduced proliferation rate compared to WT cells. E8.25 WT embryos and strong chimeras were exposed to a 1 hour pulse of BrdU to label proliferating cells. The forebrain of WT and chimeric embryos show approximately the same proportion of BrdU-incorporating cells (data not shown), indicating that there is no significant difference in the rate of division of WT and *Otx2* mutant neuroepithelial cells in the forebrain.

Tunnel labelling was also performed in WT and chimeric embryos to determine if the loss of forebrain *Otx2* mutant cells is mediated by apoptosis. Both strong and moderate chimeric embryos were analysed, at E8.5. In WT embryos, apoptotic cells were found in the dorsal midline of the neural tube and around the otic vesicle (Fig. 3A,A' and data not shown). The mutant rostral brain of strong chimeras show a dramatic increase in apoptosis (Fig. 3B,B'), which is also observed in the mutant patches and surrounding WT cells in the forebrain of moderate chimeras (Fig. 3C,C'). Therefore, the loss of the mutant cells from the forebrain in the moderate chimeras is likely due to their elimination by apoptosis.

Abnormal segregation of *Otx2* mutant cells in the midbrain

Beside the lack of mutant cells in the forebrain, there is a striking segregation between WT and *Otx2* mutant cells in the midbrain

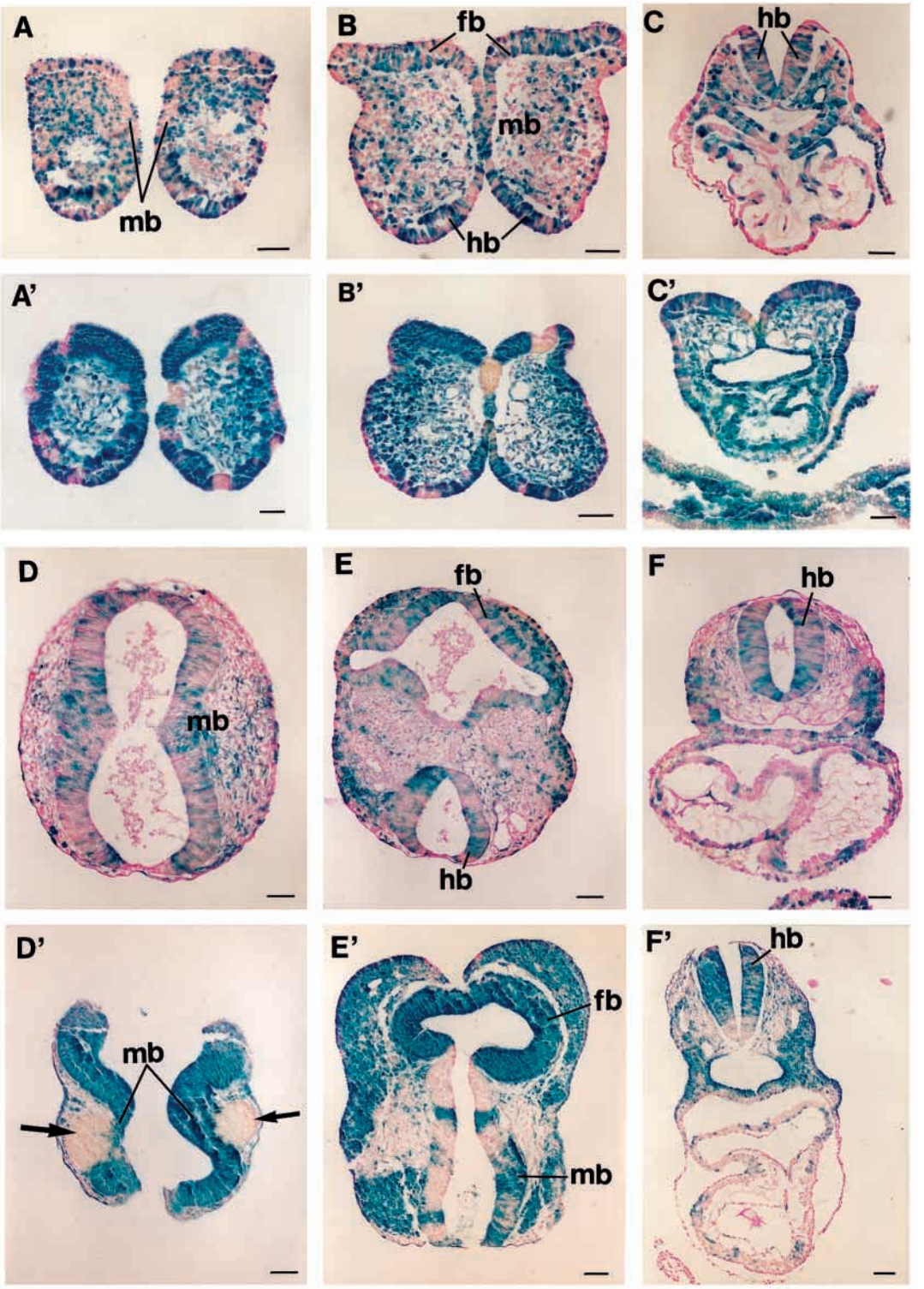

Fig. 2. *Otx2* mutant cells contribute poorly to the forebrain and segregate from WT cells in the midbrain of moderate chimeras. Transverse sections of control WT chimeras (A-F), and of moderate *Otx2* mutant chimeras (A'-F') at E8.5 (A-C, A'-C') and E9.5 (D-F, D'-F'). (A-C, A'-C') Distribution of embryonic and ES cells in moderate chimeric embryos, generated by injection of WT (A-C) and *Otx2* mutant (A'-C') ES cells into ROSA26 *lacZ+/–* embryos at E8.5 after β-galactosidase staining. *Otx2* mutant cells (pink) can contribute to the forebrain (A',B') although less efficiently than in other regions of the neural tube (A'-C'). (D-F, D'-F') By E9.5, very few or no mutant cells remain in the forebrain (E'). Moreover, mutant cells are distinctly segregated from WT cells (E') and in some cases form abnormal protrusions in the midbrain (arrows in D'). In control chimeras, WT cells derived from the embryo mix well with ES-derived cells and there is good contribution of ES-derived cells to the neural tube all along the anteroposterior axis (D-F). Abbreviations: fb, forebrain; mb, midbrain; hb, hindbrain. Scale bars, 50 μm.

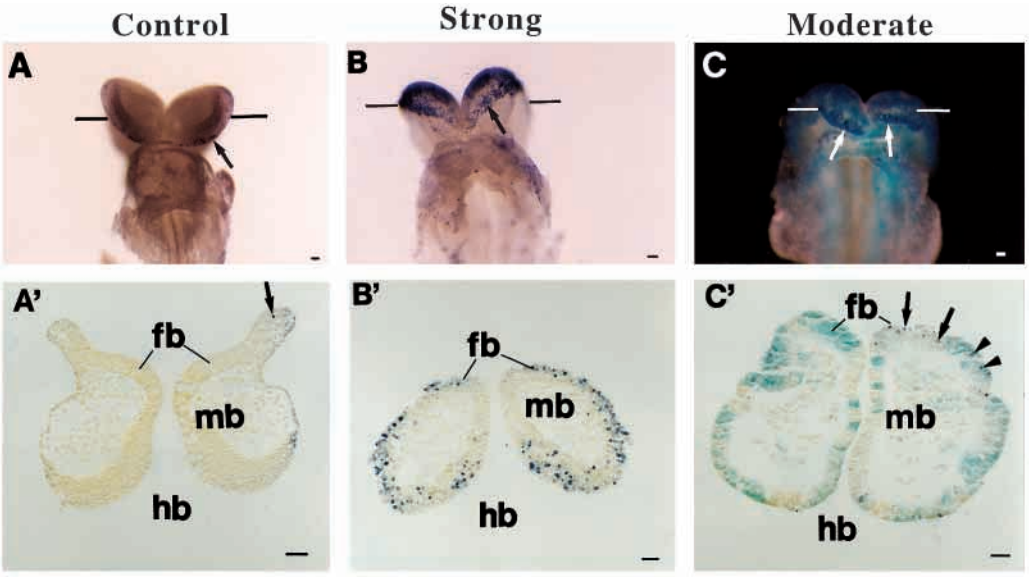

Fig. 3. *Otx2* mutant cells show high levels of apoptosis. Whole-mounts (A-C) and sections at the level of the brain (A'-C') of WT and chimeric embyos at E8.5. Both strong and moderate *Otx2–/–↔+/+* chimeras have increased numbers of apoptotic cells (arrows) in the forebrain (B,B' and C,C' respectively), compared to WT embryos (A,A'). Sections show that both *Otx2* mutant (arrows) and WT (arrowheads) cells are apoptotic in the forebrain of moderate chimeras (C'). The plane of sections is indicated by horizontal lines in the corresponding whole-mount figures. Abbreviations: same as in Fig. 2. Scale bars, 50 μm.

of moderate chimeras (*n*=7) (Fig. 2E'). Abnormal protrusions of mutant neuroepithelial cells are also visible at midbrain levels (arrow in Fig. 2D'). These protrusions occur at boundaries between mutant and WT portions of neuroepithelium and are thus likely due to an inability of mutant cells to mix with WT cells. In contrast, the degree of mixing of mutant and WT cells in more posterior regions of the neural tube of the same chimera (Fig. 2F') is similar to that observed in control chimeras at E9.5 (Fig. 2F). This lack of mixing of WT and mutant cells could be due to changes in cell adhesion of mutant cells.

Previous studies have demonstrated the existence of regulatory interactions between homeodomain-containing genes and cell adhesion molecules (reviewed by Edelman and Jones, 1998). Cadherins are a family of calcium-dependent homophilic cell surface glycoproteins which have been shown to confer differential adhesiveness to embryonic cells expressing different cadherin molecules or different levels of the same molecule in vitro (reviewed by Redies and Takeichi, 1996). The expression of two cadherins, *N-cadherin* and *R-cadherin*, has been reported in the mouse embryonic neural tube (Radice et al., 1997; Matsunami and Takeichi, 1995). We therefore examined whether the expression of these two cadherins was affected in *Otx2* mutant cells in strong and/or moderate chimeras. *N-cadherin* is normally expressed throughout the neural tube in WT embryos at E8.5 (Radice et al., 1997) and we found no change in this expression pattern in strong chimeras (*n*=2; data not shown).

We next examined expression of *R-cadherin* in strong and moderate chimeras. R-cadherin expression in restricted domains of embryonic mouse brain as early as E9.5 has been previously reported (Matsunami and Takeichi, 1995), but its expression at earlier stages has not been described. We therefore examined expression of *R-cadherin* in WT embryos at E8.5 and found that it is expressed in a broad anterior region including the forebrain and midbrain, and also in rhombomeres 1 (r1), r3 and r5 in the hindbrain (Figs 4B,B' and 5A). In strong chimeras, whose neural tube consists entirely of *Otx2* mutant cells, *R-cadherin* expression was detected at WT levels in rhombomeres 3 and 5, but appeared significantly reduced in the rostral brain (*n*=2; Fig. 5B). Reduced levels of *R-cadherin* in *Otx2* mutant cells was confirmed in moderate chimeras. *R-cadherin* was expressed in the forebrain and midbrain of moderate chimeras, but *R-cadherin* transcripts levels appeared uneven (*n*=2; Fig. 5C), compared to WT embryos (Figs 4B' and 5A). Lateral views of the the forebrain and midbrain showed that this patchy appearance was due to the presence of groups of cells with lower levels of *R-cadherin* expression surrounded by cells expressing WT levels of *R-cadherin* (Fig. 5D,E). Double-labelling experiments for β-galactosidase activity and *R-cadherin* expression were not attempted because β-galactosidase staining would mask the quantitative differences in *R-cadherin* expression. However, the previously reported observations of a segregation between mutant and WT cells in moderate chimeras (Figs 1C and 2D',E'), and of a reduction of *R-cadherin* transcripts levels in mutant cells of strong chimeras (Fig. 5B) suggest that the patches with lower levels of *R-cadherin* expression correspond to groups of *Otx2* mutant cells surrounded by WT cells. These data therefore suggest that *Otx2* regulates the expression of *R-cadherin* in cells of the rostral brain.

Other cell surface molecules that have been implicated in regulating adhesive interactions of neuroepithelial cells are members of the Eph receptor tyrosine kinases and their ligands called ephrins (reviewed by Flanagan and Vanderhaeghen, 1998). In particular, microinjection of a dominant negative form of the Eph receptor, *Sek1* into *Xenopus* and zebrafish embryos leads to disruption of the spatial restriction of r3/r5 gene expression in the hindbrain (Xu et al., 1995). *ephrin-A2* is expressed in the midbrain of mouse embryos starting at E8.5 (Cheng et al., 1994). In wild-type embryos and control chimeras, we found that its posterior boundary of expression in the midbrain coincides with that of *Otx2* at this stage (Figs 4C,C' and 6A). The similarity of their expression domains in the midbrain suggested that *Otx2* may regulate expression of *ephrin-A2*. We therefore examined its expression in *Otx2*-mutant cells in moderate chimeras. Interestingly, we found that expression of *ephrin-A2* is missing in patches of mutant cells in the midbrain (*n*=2; Fig. 6B,C), demonstrating that *Otx2* regulates expression of *ephrin-A2* cell-autonomously in this brain region.

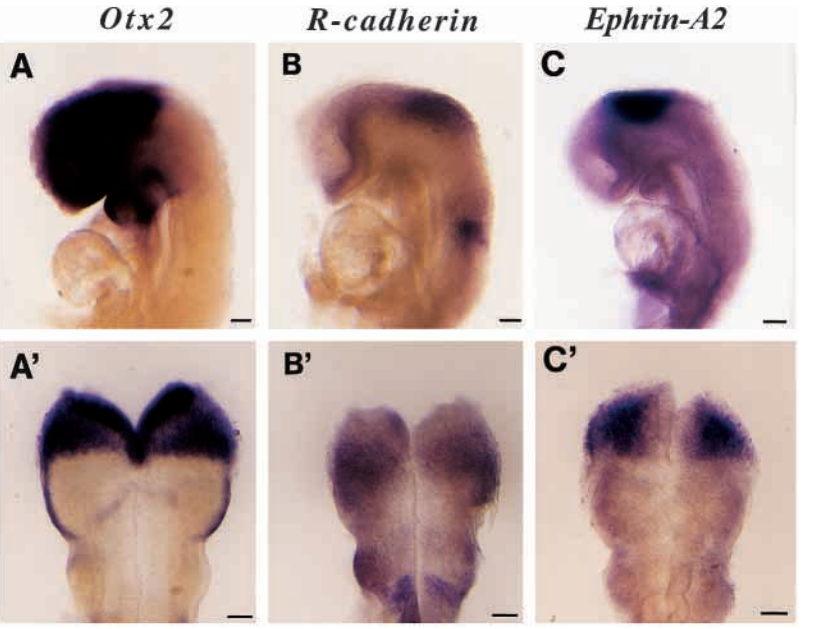

Fig. 4. *R-cadherin* and *ephrin-A2* are co-expressed with *Otx2* in the midbrain. (A-C) Lateral views and (A'-C') dorsal views of flatmounts of E8.5 embryos. (A,A') Wild-type expression of *Otx2* in the forebrain and midbrain. (B,B') *R-cadherin* expression in the forebrain and midbrain overlaps with that of *Otx2*. In addition, *R-cadherin* is also expressed in r1, r3 and r5 of the hindbrain. (C,C') The posterior boundary of *ephrin-A2* in the midbrain is similar to that of *Otx2* (A'). Scale bars, 100 μm.

Cell autonomous and non-cell autonomous requirements for *Otx2* in regulating the expression of regional markers of the rostral brain

We have previously shown that the initiation and/or maintenance of expression of several forebrain and midbrain regional genes is affected in the *Otx2* mutant cells of strong chimeras (Rhinn et al., 1998). In the forebrain, *Otx2* is required to activate the expression of the homeobox gene *Rpx* and maintain the expression of another homeobox gene, *Six3*. To determine if *Otx2* is required cell autonomously or non-cell autonomously to regulate expression of these genes, we analysed the forebrain of moderate chimeric embryos in double-labelling experiments, using histochemical staining for β-galactosidase activity to distinguish WT from *Otx2* mutant cells, and whole-mount RNA in situ hybridization to characterise *Rpx* or *Six3* expression. *Rpx* is expressed in the forebrain of control embryos at E8.5 (Fig. 7A and Thomas and Beddington, 1996; Hermesz et al., 1996). In moderate chimeras, *Rpx* expression was absent from the patches of *Otx2* mutant cells, but was present in the surrounding WT forebrain cells (*n*=5; Fig. 7B-D). At the border of the mutant cell patches, *Otx2* mutant cells failed to express *Rpx* while neighbouring WT forebrain cells maintained expression of the gene (Fig. 7D). The strict correlation at the cellular level between lack of *Otx2* activity and loss of *Rpx* expression demonstrates that *Otx2* is required cell autonomously for expression of this gene in the forebrain. In contrast, *Six3*, another homeobox gene expressed in the forebrain (Oliver et al., 1995 and Fig. 7E), was expressed in groups of *Otx2* mutant cells as in surrounding WT cells in moderate chimeras at E8.5 (*n*=2; Fig. 7F,G), indicating that *Otx2* is required non-cell autonomously for maintenance of *Six3* expression. Thus, *Otx2* regulates expression of different regulatory genes in the forebrain through distinct pathways.

Similar results were obtained for the regulation of gene expression in the mid-hindbrain region. *Otx2* is required for the activation of expression of the signalling molecule *Wnt1* and for the maintenance of expression of the homeobox gene *En2* (Rhinn et al., 1998). *Wnt1* expression was observed in WT midbrain cells in control embryos and moderate chimeras but was not detected in any *Otx2* mutant (white) cells in the midbrain of moderate chimeras, including those in contact with WT cells (*n*=2; Fig. 8A-D). This result demonstrates that *Otx2*

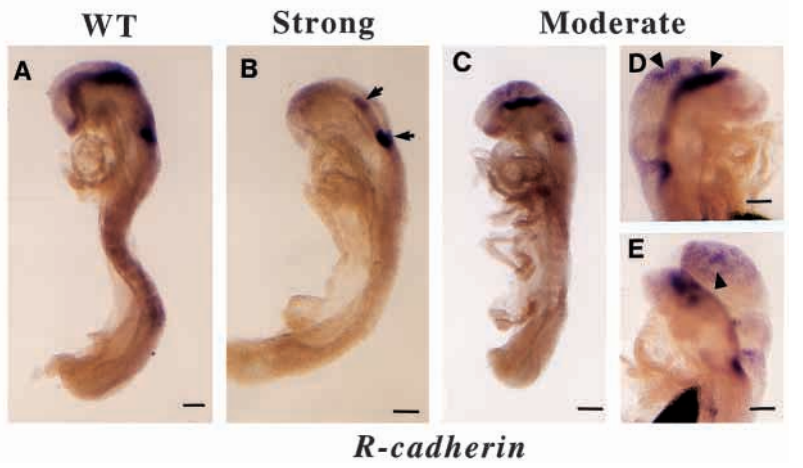

Fig. 5. *R-cadherin* expression is reduced in strong and moderate *Otx2* mutant chimeras. (A,B) *R-cadherin* expression in a WT embryo (A) and a strong chimera (B) at E8.5. *R-cadherin* is normally expressed in the forebrain, midbrain and in r1, r3 and r5 in the hindbrain (A). In strong chimeras, expression of *R-cadherin* is severely reduced in the rostral brain (forebrain, midbrain and r1) (B), while its expression in r3 and r5 (arrows in B) is similar to WT embryos. (C-E) *R-cadherin* expression in moderate *Otx2–/–↔+/+* chimeras. Expression of *R-cadherin* is patchy (arrowheads in D and E), presumably due to the lower levels of *R-cadherin* in *Otx2* mutant cells in the rostral brain. Scale bars 100 μm.

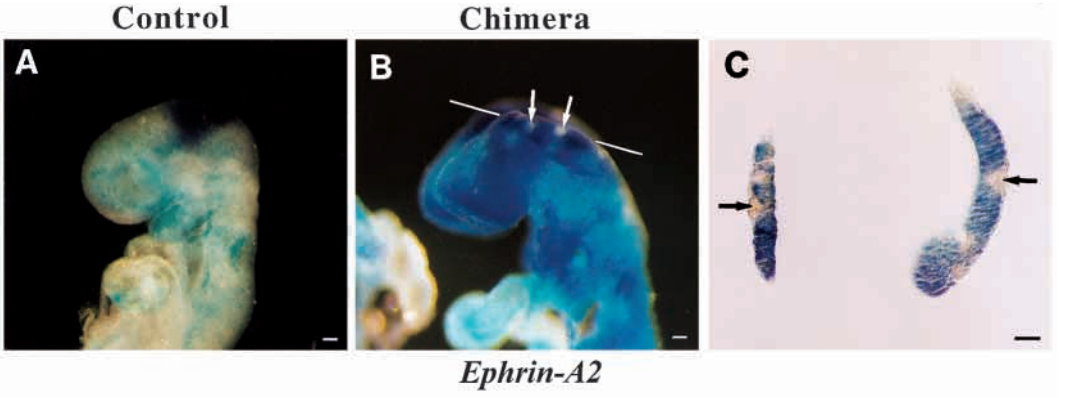

Fig. 6. *Otx2* is required cell autonomously for expression of *ephrin-A2*. (A) A control chimeric embryo showing expression of *ephrin-A2* in the midbrain. (B,C) In moderate chimeras, *ephrin-A2* is expressed in wild-type cells (dark blue) but not in *Otx2* mutant cells (white, arrows) in the midbrain. The plane of the section shown in C is indicated by white bars in B. Scale bars, 100 μm.

is required cell autonomously in midbrain cells to activate *Wnt1* expression. In contrast, *En2* expression in *Otx2* mutant cells in the mid-hindbrain of moderate chimeras was rescued by the presence of surrounding WT cells (*n*=5; Fig. 8E-G), demonstrating a non-cell autonomous function for *Otx2* in regulating *En2* expression. Therefore, *Otx2* also regulates the expression of mid-hindbrain genes through different mechanisms.

DISCUSSION

We have characterised the phenotype of *Otx2* mutant cells at the cellular level in *Otx2*–/–↔+/+ chimeras. These studies demonstrate that *Otx2* is required in the neurectoderm both for specification of forebrain and midbrain territories and for the control of brain morphogenesis through regulation of cell surface properties. In addition, we have identified several putative transcriptional targets of *Otx2* that could mediate its activity in these processes.

Otx2 is required for the maintenance of forebrain but not midbrain cells

There is a strong selection against *Otx2* mutant cells in the forebrain, as mutant cells are still present in the forebrain of moderate chimeras at E8.5, but are absent at E9.5. The progressive loss of mutant cells from the forebrain could either be due to their elimination by apoptosis or to defects

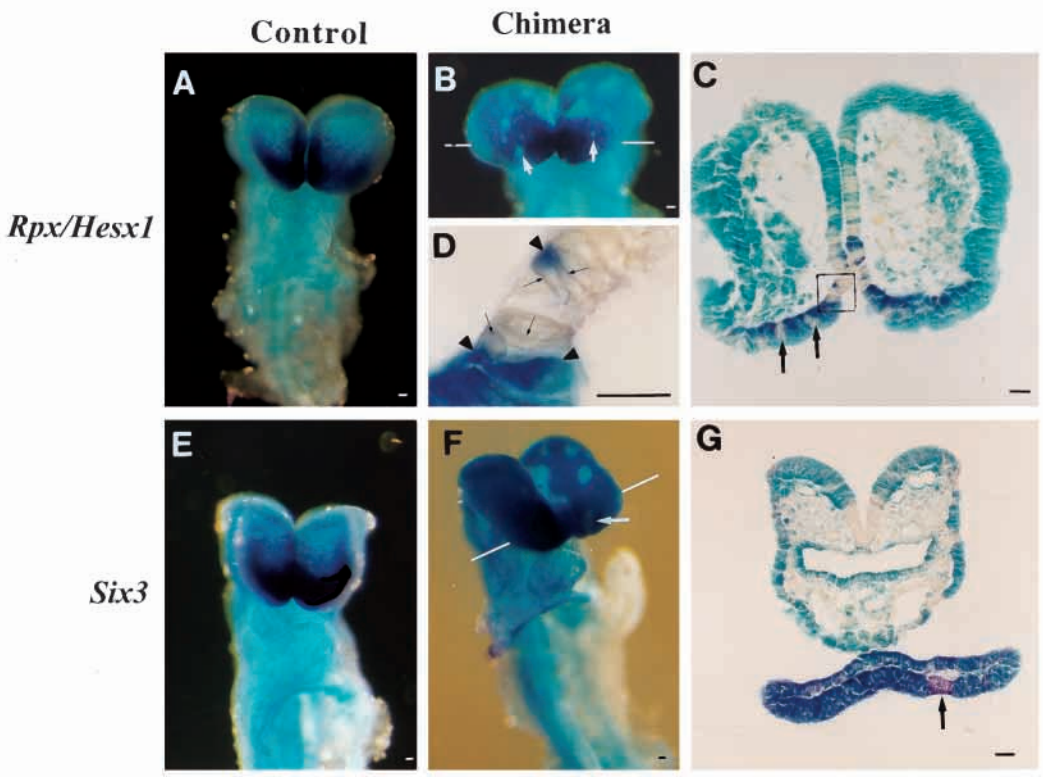

Fig. 7. *Otx2* is required cell autonomously for *Rpx/Hesx1* expression and non-cell autonomously for *Six3* expression in the forebrain. (A,E) Control chimeric embryos and (B,C,D,F,G) moderate chimeras. (A-D) *Rpx/Hesx1* expression at E8.5. (A,B) *Rpx/Hesx1* expression is missing in patches of *Otx2* mutant cells (arrows in B) in the ventral forebrain. (C) Section through the forebrain of the chimera in B showing that *Rpx/Hesx1* expression (dark blue) is lacking in *Otx2* mutant cells (white, arrow), in contrast to its expression in WT cells of the ventral forebrain. The plane of section is shown by white bars in B. (D) Higher magnification of the boxed region in C showing that *Otx2* mutant cells (arrows) at the WT/mutant cell boundaries also do not express *Rpx*, in contrast to wild-type cells (arrowheads). (E-G) *Six3* expression at E8.5. (E,F) *Six3* is expressed in *Otx2* mutant cells (arrow in F) and WT cells in the ventral forebrain. Patches of mutant cells in the midbrain of this chimeric embryo (outside of the *Six3* expression domain) appear lighter blue (due to *lacZ* expression of underlying tissues) than the deep blue of the surrounding *lacZ*+ WT cells. (G) Section through the forebrain of the chimera shown in F showing that *Six3* is expressed in a patch of *Otx2* mutant cells (purple, arrow) in the ventral forebrain. The plane of the section is indicated by white bars in F. Scale bars, 25 μm.

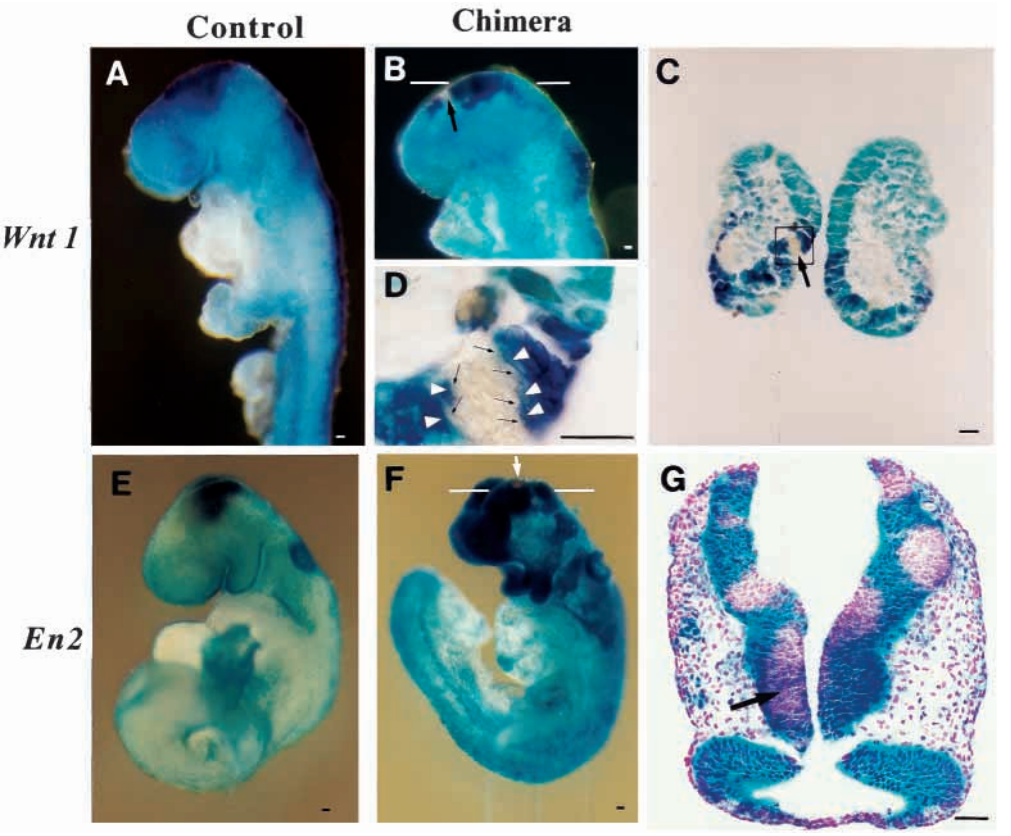

Fig. 8. *Otx2* is required cell autonomously for *Wnt1* expression and non-cell autonomously for *En2* expression in the mid-hindbrain region. (A,E) WT embryos and (B,C,D,F,G) moderate chimeras. (A-D) *Wnt1* expression at E8.5. (A,B) *Wnt1* expression is missing in *Otx2* mutant cells (white, arrow in B), in contrast to its expression in WT cells (dark blue) in the midbrain. (C) Section of the chimera shown in B showing the absence of *Wnt1* expression in *Otx2* mutant cells (white, arrow) in the midbrain. The plane of the section is indicated by white bars in B. (D) Higher magnification of the boxed region in C showing that *Otx2* mutant cells (arrows) at the WT/mutant cell boundaries also do not express *Wnt1*, in contrast to the WT cells (arrowheads). (E-G) *En2* expression at E8.75. (E,F) *En2* is expressed in *Otx2* mutant (arrow in F) and WT cells in the mid-hindbrain region. (G) Section of the chimera shown in F showing that *En2* is expressed in *Otx2* mutant cells (purple, arrow) in the midbrain and anterior hindbrain. The plane of the section is indicated by white bars in E. Scale bars, 25 μm.

in cell proliferation, as a result of improper specification. No difference in proliferation rate was observed between WT and mutant cells in the forebrain of chimeras. In contrast, TUNEL analysis indicates that mutant cells in the forebrain of strong and moderate chimeras show a higher rate of apoptosis than forebrain cells in WT embryos. Thus, the defect in specification of *Otx2* mutant cells in moderate chimeras, demonstrated by their failure to activate the expression of important regulatory genes such as *Rpx* and *Wnt1*, rapidly results in apoptotic death**.** In contrast, mutant cells remain in the midbrain of chimeric embryos at E9.5, demonstrating a more stringent requirement for *Otx2* function in the forebrain than in the midbrain. The death of forebrain WT cells in moderate chimeras could be due to a toxic effect of the dying mutant cells, or a more specific non-cell autonomous function of *Otx2* in the maintenance of forebrain cells.

Cell autonomous and non-cell autonomous regulation of target genes by *Otx2*

The cell autonomous requirement for *Otx2* function in *Rpx* expression in the forebrain and *Wnt1* expression in the midbrain suggest that *Otx2* may directly regulate the transcription of these genes by binding to promoter sequences. A 110 bp *Wnt1* enhancer fragment has been shown to be sufficient to confer early midbrain-specific expression to a *lacZ* reporter gene in mouse embryos (Rowitch et al., 1998). This enhancer contains several homeobox consensus binding sites and it will be important to determine whether *Otx2* binds specifically to these sequences. Although *Otx2* is required for expression of *Wnt1* in the midbrain, it is certainly not sufficient since *Otx2* expression domain is broader than that of *Wnt1*. We therefore propose that *Otx2* functions together with coregulators to regulate expression of *Wnt1*, perhaps through synergistic interactions with LIM domain-containing cofactors, as previously shown for the transcriptional activity of a pituitary *Otx* class homeodomain protein, POTX/Ptx1 (Bach et al., 1997). Similarly, *Otx2* could directly regulate the expression of *Rpx* in the forebrain, through binding to *bicoid* class recognition sequences that are present in the *Rpx* promoter (Kathy Mahon, personal communication). In vitro binding studies and co-transfection experiments with reporter constructs in cell cultures will be required to further test these hypotheses of direct interactions.

In contrast, expression of the genes *Six3*, in the forebrain, and *En2*, in the mid-hindbrain, require *Otx2* non-cell autonomously. In these cases, regulation by *Otx2* must be indirect and include a step of cell-cell signalling. In particular, as *Wnt1* has been shown to regulate *En1* and *En2* expression in the midbrain (Danielian et al., 1996), it is likely that the loss of *En2* expression in strong *Otx2* chimeras is due to the complete lack of midbrain *Wnt1* expression in these embryos, and that the rescue of *En2* expression in *Otx2* mutant cells of moderate chimeras is due to the production by surrounding WT cells, and paracrine activity, of WNT1. In addition, FGF8, which has been shown to be capable of inducing En genes when supplied ectopically via beads (Crossley et al., 1996) could also be involved as well as other yet unidentified secreted molecules. There is currently no good candidate signalling molecule to control *Six3* expression in the forebrain.

Cell adhesion defects in the *Otx2* mutant brain

Otx2 mutant cells segregate from WT cells in the midbrain neuroepithelium of moderate chimeras. This defect is likely to arise from changes in cell adhesive properties of mutant cells. Recent studies using mutant mice and in vitro cell sorting assays have demonstrated a link between regional specification and cell surface properties in the forebrain (Götz et al., 1996; Stoykova et al., 1997) and in the hindbrain (Wizenmann and Lumsden, 1997). In particular, the studies in the forebrain have suggested that the paired and homeobox-containing gene *Pax6* regulates expression of *R-cadherin* and is involved in specifiying the cortico-striatal boundary. These data, together with the published expression pattern of *R-cadherin* in specific neuromeres of the mouse embryonic brain and the segregation of R-cadherin-positive from R-cadherin-negative cells in vitro (Matsunami and Takeichi, 1995) lead to us to investigate whether *R-cadherin* expression was affected in *Otx2* mutant neuroepithelial cells. We observed that expression of *R-cadherin* is first initiated in the midbrain at E8.25 (data not shown), and that by E8.5, it is expressed in the forebrain, the midbrain and rhombomeres one, three and five in the hindbrain. The study of chimeric embryos suggest that *Otx2* regulates the expression of *R-cadherin*, although the gene is still expressed at reduced levels in absence of *Otx2* activity.

More strickingly, we have found that *Otx2* also regulates expression of *ephrin-A2* in a cell-autonomous manner. In vivo blocking experiments have suggested that the restriction of neuroepithelial cell intermingling between adjacent rhombomeres (Xu et al., 1995) and between different regions of the diencephalon (Xu et al., 1996) requires interactions between Eph receptors and ephrin ligands. Thus, segregation of *Otx2* mutant from wild-type cells could be due to differences in their expression of ephrin ligands, such as *ephrin-A2*, and possibly also of cognate Eph receptors. Future examination of the role of Otx2 in cell adhesion in the midbrain, and the involvement of ephrin-Eph receptor interactions in this process, will require the use of in vitro cell sorting assays and blocking reagents.

Establishment of organising centers in the brain

The ability of *Otx2*-expressing cells to segregate from *Otx2*-negative cells in the brain may be instrumental in vivo to prevent mixing between mesencephalic and metencephalic cells. This mechanism may be important for the formation of a sharp mes-met boundary, which is critical for the establishment of the isthmic organizer. A role for *Otx2* in establishment of the isthmic organiser is supported by genetic manipulation experiments in mice (Acampora et al., 1997; Suda et al., 1997). A recent study in zebrafish has suggested the presence of another organising center in the forebrain (Houart et al., 1998). It is tempting to speculate that the rostral boundary of *Otx2* expression in the forebrain may be similarly involved in the establishment of an organising center in this region.

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