Development 126, 743-757 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV2363

Functional equivalency between *Otx2* and *Otx1* in development of the rostral head

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Accepted 27 November 1998; published on WWW 20 January 1999

SUMMARY

Mice have two *Otx* genes, *Otx1* and *Otx2*. Prior to gastrulation, *Otx2* is expressed in the epiblast and visceral endoderm. As the primitive streak forms, *Otx2* expression is restricted to the anterior parts of all three germ layers. *Otx1* expression begins at the 1 to 3 somite stage in the anterior neuroectoderm. *Otx2* is also expressed in cephalic mesenchyme. *Otx2* homozygous mutants fail to develop structures anterior to rhombomere 3 (r3), and *Otx2* heterozygotes exhibit craniofacial defects. *Otx1* homozygous mutants do not show apparent defects in early brain development. In *Otx1* and *Otx2* double heterozygotes, rostral neuroectoderm is induced normally, but development of the mes/diencephalic domain is impaired starting at around the 3 to 6 somite stage, suggesting cooperative interactions between the two genes in brain regionalization. To determine whether *Otx1* and *Otx2* genes are functionally equivalent, we generated knock-in mice in which *Otx2* was replaced by *Otx1*. In homozygous mutants, gastrulation occurred normally, and rostral neuroectoderm was induced at 7.5 days postcoitus (7.5 dpc), but the rostral brain failed to develop. Anterior structures such as eyes and the anterior neural ridge were lost by 8.5 dpc, but the isthmus and r1 and r2 were formed. In regionalization of the rostral neuroectoderm, the cooperative interaction of *Otx2* with *Otx1* revealed by the phenotype of *Otx2* and *Otx1* double heterozygotes was substitutable by *Otx1*. The otocephalic phenotype indicative of *Otx2* haploinsufficiency was also largely restored by knocked-in *Otx1*. Thus most *Otx2* functions were replaceable by *Otx1*, but the requirement for *Otx2* in the anterior neuroectoderm prior to onset of *Otx1* expression was not. These data indicate that *Otx2* may have evolved new functions required for establishment of anterior neuroectoderm that *Otx1* cannot perform.

Key words: Head organizer, *Otx2*, *Otx1*, Visceral endoderm, Rostral brain induction, Brain regionalization, Cephalic neural crest cells, Isthmus, Knock-in, Homeobox, Mouse

INTRODUCTION

The vertebrate head is a unique structure; no comparable structure is seen in cephalochordates or urochordates (Balfour, 1881; Goodrich, 1930; DeBeer, 1937; Northcutt and Gans, 1983; Bulter and Hoods, 1996; Janvier, 1996; Nieuwenhuys, 1977). How head development and regionalization occur is an area of intense investigation in developmental biology. Both processes start at gastrulation, and Spemann and Mangold distinguished the head organizer, which induces the anterior part of the body, from the trunk organizer (1924). In mice, the visceral endoderm is now being investigated as the site of head organizer activity (Thomas and Beddington, 1996; Varlet et al., 1997; Belo et al., 1997; Beddington and Robertson, 1998; Biben et al., 1998; Shwalot et al., 1998; Thomas et al., 1998; Waldrip et al., 1998).

As gastrulation proceeds the region of the rostral head is determined throughout the three germ layers. Roles for prechordal mesendoderm in patterning of the rostral brain have been suggested (Nieuwkoop, 1950; Toivonen and Saxen, 1968; Ang et al., 1994; Ang and Rossant, 1994; Weinstein et al., 1994; Chiang et al., 1996; Foley et al., 1997; Dale et al., 1997). The isthmus and anterior neural ridge have been proposed as centers involved in anterior/posterior patterning in the midbrain and forebrain, respectively (Marin and Puelles, 1994; Crossley and Martin, 1995; Crossley et al., 1996; Joyner et al., 1996; Shimamura and Rubenstein, 1997; Houart et al., 1998). Regionalization in the rostral brain generates telencephalon, diencephalon, mesencephalon and the isthmus, followed by further subdivisions (Puelles and Rubenstein, 1993; Rubenstein et al., 1994). Cephalic neural crest cells also play essential roles in head development; they form structures such as anterior cranium and cranial nerves (Northcutt and Gans, 1983; Matsuo et al., 1995). Recently, several genes have been proposed to play critical roles in the processes of head development. *Otx2*, a *bicoid*-class homeobox gene, is one such gene.

Otx2 is first expressed in the epiblast (Simeone et al.,

1993). Before gastrulation it is also expressed in the anterior visceral endoderm (Acampora et al., 1995). *Otx2* homozygous mutants fail to develop structures anterior to rhombomere 3 (r3), as do *Lim1* mutants (Acampora et al., 1995; Matsuo et al., 1995; Shawlot and Behringer, 1995; Ang et al., 1996). Subsequently, *Otx2* is expressed in anterior parts of all three germ layers. *Otx1* expression follows in the anterior neuroectoderm, and the caudal limit of expressions of both *Otx* genes in the neuroectoderm coincides with the boundary between the midbrain and hindbrain. *Emx2* and *Emx1* are then expressed in a more limited region. The expression patterns of *Otx* and *Emx* genes suggest a combinatorial code for rostral brain development, analogous to the *Hox* code for hindbrain development (Simeone et al., 1992). Cooperative functions between *Otx1* and *Otx2* genes in the formation of mesencephalon and caudal diencephalon were demonstrated by *Otx1* and *Otx2* double mutants (Suda et al., 1996, 1997; Acampora et al., 1997). The *Otx2* gene also cooperates with the *Emx2* gene in development of the telencephalon and anterior diencephalon (Yoshida et al., 1997; our unpublished result). In addition, *Otx2* is expressed in cephalic neural crest cells (Kimura et al., 1997), and *Otx2* heterozygotes exhibit otocephaly due to defects in these cells (Matsuo et al., 1995).

All gnathostomes have two lineages of *Otx* cognates: *Otx1* and *Otx2*. The *Otx2* cognates are highly conserved, while *Otx1* cognates are relatively diverged in gnathostomes. *Xenopus* and zebrafish, in which polyploidization took place independently, have a third cognate which apparently belongs to the *Otx1* lineage (Mori et al., 1994; Mercier et al., 1995; Kablar et al., 1996; Ueki et al., 1998). In an agnathous vertebrate, the freshwater lamprey, we have also identified two *Otx* cognates, *LjOtxA* and *LjOtxB* (Ueki et al., 1998). In contrast, *Amphioxus* and ascidians, sister groups of vertebrates, appear to have only one *Otx* gene (Wada et al., 1996; Williams and Holland, 1998). There are structural differences between vertebrate and invertebrate *Otx* cognates (Freund et al., 1997; Furukawa et al., 1997; Ueki et al., 1998). It is tempting to speculate that *Otx* gene duplication contributed to, or permitted, the evolution of the rostral brain in vertebrates and that each gene recruited different downstream targets. In mice, however, the head develops apparently normally with only one *Otx* gene, *Otx2*: *Otx1* null mutants exhibit brain defects only later in neurogenesis (Acampora et al., 1996; Suda et al., 1996, 1997). In lamprey, *LjOtxB* is not expressed in rostral neuroectoderm, and rostral brain expresses only one gene, *LjOtxA* (Ueki et al., 1998). Thus, it is probable that the vertebrate brain evolved with only one *Otx* gene in ancestral agnatha and that *Otx* gene duplication took place independently in lineages of the extant agnatha and gnathostomes.

In the gnathostome lineage, it is *Otx2* cognates in tetrapods but *Otx1* in zebrafish that are first expressed in early gastrulation and during early anterior neurulation (Simeone et al., 1993; Li et al., 1994; Mori et al., 1994; Bally-Cuif et al., 1995; Mercier et al., 1995; Pannese et al., 1995; Kablar et al., 1996; Ueki et al., 1998). It was recently reported that human *OTX1* and *OTX2* can substitute for functions of *orthodenticle* (*otd*), the *Drosophila* orthologue of *Otx* (Finkelstein et al., 1990), in the fruit fly (Leuzinger et al., 1998; Nagao et al., 1998). Likewise, *otd* can substitute for *Otx1* in mice (Acampora et al., 1998). To address the question of whether *Otx1* is functionally equivalent to *Otx2*, we generated mice in which *Otx2* was replaced with *Otx1*. Analysis of the knock-in mice showed that most *Otx2* functions are replaceable with those of *Otx1*, but that those in the anterior neuroectoderm prior to the onset of the *Otx1* expression are not.

MATERIALS AND METHODS

Construction of targeting vectors

Mouse *Otx1* and *Otx2* cDNAs were isolated from a mouse 11 dpc cDNA library (Clontech); the nucleotide sequences were confirmed by standard sequencing methods. To construct the replacement vector, the *Eco*RI-*Bam*HI fragment of pKJ2 (Boer et al., 1990) containing the neo cassette (the neo resistance gene driven by the phosphoglycerate kinase 1 (*PGK1*) promoter and lacking a polyadenylation signal) was inserted into *Bam*HI and *Hin*dIII sites between two loxP sites of pBS246 (Gibco BRL) by blunt end ligation. A fusion gene consisting of the 5′ non-coding region of *Otx2* cDNA, the coding region of *Otx1* cDNA, the 3′ non-coding region of *Otx2* cDNA, the SV40 t splice site and the SV40 polyadenylation signal was constructed (Fig. 1B) and flanked by *Sma*I and *Eco*RV sites at the 5′ and 3′ ends, respectively. The *Sma*I/*Eco*RV fragment was fused with the *Eco*RV/*Sca*I fragment of the neo cassette flanked with loxP. The resulting fusion product was inserted between two *Sma*I sites that flank the translation initiation codon of *Otx2*, one located 213 bp upstream and the other 21 bp downstream of the ATG. The length of the homologous region was 6.7 kb and 4.1 kb at the 5′ and 3′ sides of the insert, respectively (Fig. 1A). The targeting vector contained a diphtheria toxin A fragment (DT-A) gene driven by the MC1 promoter for negative selection as described by Yagi et al. (1993b). A targeting vector for the *lacZ* knock-in was constructed using the same strategy. Details of the construction of the targeting vectors are available upon request.

Generation of knock-in mice

TT2 ES cells (Yagi et al., 1993a) were cultured, electroporated with the *Sal*I-linearized targeting vector and selected in G418 as described by Nada et al. (1993); Yagi et al. (1993b). Homologous recombinants were obtained at a frequency of 11/191 and of 5/139 G418 resistant clones for *Otx1* and *lacZ* knock-ins, respectively.

Chimeric mice were obtained by injecting homologously recombined TT2 cells into 8 cell embryos as described by Yagi et al. (1993a). Male chimeras were mated with C57BL/6 females. The resulting zygotes were injected with the *Cre* gene driven by the chicken β-actin promoter to delete the loxP-flanked neo cassette, as described by Araki et al. (1995). *Otx1* and *Otx2* mutants were generated previously (Matsuo et al., 1995; Suda et al., 1996). The genetic background of mice used in the present study is described in the Results. The mice were housed in environmentally controlled rooms of the Laboratory Animal Research Center of Kumamoto University School of Medicine under University guidelines for animal and recombinant DNA experiments.

Genotyping

Genotypes of newborn mice and embryos were routinely determined by PCR (polymerase chain reaction) analyses and confirmed, when necessary, by Southern blots of genomic DNAs prepared from tails or yolk sacs. In the PCR analyses, the primers and the length of the product were as follows: wild-type *Otx2* allele (305 bp) with primer OX24 (5′-TAGTTAGTGGAACGTGGAGGAAGCTGC) and primer p104 (5′-ATACATCGGGAACCCCGCCTTGAGCTTGAG); *Otx1* knock-in *Otx2* allele (728 bp) with primer SV2 (5′-GCCTTGACTAGAGATCATAATCAGCCA) and primer p104; *lacZ* knock-in *Otx2* allele (600 bp) with primer N3 (5′-GCCTGCTTGCCGAATATCATGGTGGAAAAT) and primer p105 (5′-AATGCTCTGTGGCACTCGGCAGTTTGGTAG). The *Otx2* and

Otx1 null alleles were identified as described by Matsuo et al. (1995); Suda et al. (1996). PCR analyses to discriminate the *Otx2* allele from CBA and C57BL/6 mice were performed using primers pM1 (5′-GAGAACTCAGTCTTGTATCCG) and pM2 (5′-GGCAAT-GTGTGTAAGGCTGG) (Oyanagi et al., 1997).

In situ hybridization

Embryos were dissected in PBS and fixed overnight at 4°C in 4% paraformaldehyde (PFA) in PBS. Embryos were gradually dehydrated in methanol/PBT (PBS-0.1% Tween-20) up to 100% methanol and stored at −20°C. The protocol for whole mount in situ hybridization of embryos was as described by Wilkinson (1993), using single-stranded digoxigenin-UTP labeled RNA probes (Boehringer Mannheim). The probes used were those described for *Wnt1* (McMahon and Bradley, 1990), *En1* (Davis and Joyner, 1988), *Pax2* (Dressler et al., 1990), *Fgf8* (Crossley and Martin, 1995), *Emx2* (Yoshida et al., 1997), *Otx1* and *Otx2* (Matsuo et al., 1995), *Krox20* (Wilkinson et al., 1989), *Six3* (Oliver et al., 1995), *Hesx1/Rpx* (Hermesz et al., 1996), *Fkh2* (Kaestner et al., 1995), *HNF3β* (Sasaki and Hogan, 1993), *Brachyury* (Herrmann, 1991) and *cer-l* (Belo et al., 1997).

RT-PCR analysis

RT (reverse transcription)-PCR analyses were performed with total RNAs isolated from 7.5 dpc embryos and reverse-transcribed with oligo(dT)$_{17}$ (Ilic et al., 1995). The primers used for the *Otx1* expression were p1 (5′-ATGATGTCTTACCTCAAACAACCC) from the first exon and p2 (5′-TGAGCGCGTGAAGGTGGTGCGCTC) from the second exon. Those for *Otx2* expression were RT1 (5′-TCTTATCTAAAGCAACCGCCTTACGCAGTC) from the first exon and OX2A (5′-GCACCCTGGATTCTGGCAAGTTGATTTTCA) from the second exon.

β-Gal staining

Embryos were fixed in 1% formaldehyde, 0.2% glutaraldehyde and 0.02% NP-40 in PBS for 10 minutes, followed by three washes with PBS for 10 minutes at room temperature. Staining was carried out overnight at 37°C in PBS containing 1 mg/ml X-Gal, 20 mM $K_3Fe(CN)_6$, 20 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, and 0.02% NP-40. Stained embryos were washed twice with PBS and immediately stored in 10% formaldehyde.

Western blot analysis

A rabbit anti-mouse Otx1 peptide (GGSYGQGYPAPSSSY) polyclonal serum was generated by standard procedures (Research Genetics) and purified twice on a protein A sepharose (Amersham Pharmacia) and on an affinity column of Otx1 peptide immobilized on FMP-activated cellulofine (Seikagaku Kogyo).

Embryos were homogenized in suspension buffer (0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.5, 100 μg/ml PMSF and 1 μg/ml aprotinine). The homogenates were combined with an equal volume of loading buffer (100 mM Tris-HCl pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue and 20% glycerol), denatured at 100°C for 10 minutes, subjected to 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The membrane was probed with the affinity purified anti-Otx1 antibody and reprobed with peroxidase-conjugated second antibody. Immunoreactive proteins were visualized with the Enhanced Chemiluminescence Detection System (Amersham) on X-ray film.

Skeletal analysis and histology

Cartilage and bones were stained with alcian blue and alizarin red by the method of Kelly and Bryden (1983) and stored in 80% glycerol. For histological analyses, mouse embryos were fixed with Bouin's fixative solution, dehydrated and embedded in paraplast. Serial sections (8 μm thick) were made and stained with 0.1% cresyl violet (Sigma) or with Hematoxylin and Eosin. Cranial nerve morphology was examined with the monoclonal antibody 2H3 by whole-mount immunostaining procedures (Sundin and Eichele, 1990; Matsuo et al., 1995).

RESULTS

Otx1 knock-in into *Otx2* locus

Fig. 1A shows the targeting vector used to knock *Otx1* into the *Otx2* locus. Since the transcriptional regulatory elements of the *Otx2* gene are not yet fully characterized (Kimura et al., 1997), *Otx1* cDNA was inserted at the translation start site of the *Otx2* gene to minimize deletion of *Otx2* sequences (see Materials and Methods). The *Otx1* cDNA contained two polyadenylation signals, one derived from *Otx2* and the other from SV40 early genes, followed by the neomycin resistance gene driven by the *PGK1* promoter and lacking a polyadenylation signal (neo cassette). To avoid potential interference with *Otx2* transcriptional regulatory elements by the *PGK1* promoter, the neo cassette was flanked with loxP sites for deletion with Cre recombinase. Since it was possible that differences in non-coding sequences in *Otx2* and *Otx1* cDNAs might affect RNA stability or translational regulation, all non-coding sequences of *Otx1* cDNA were replaced with those from *Otx2* cDNA, as shown in Fig. 1B.

Homologous recombinants were obtained using TT2 ES cells derived from an F_1 embryo from C57BL/6xCBA mice. To delete the neo casette, zygotes obtained from crosses of male chimeras with C57BL/6 females were injected with the *Cre* gene under control of the chicken β-actin promoter (Araki et al., 1995) and then allowed to develop in foster mothers to generate F_1 offspring. F_1 mice in which the deletion of the neo cassette was confirmed (Fig. 1C) were crossed with C57BL/6 to analyze the heterozygous phenotype. The homozygous phenotype was examined in the F_3 generation following crosses among F_2 heterozygous knock-in mice. Two independent knock-in mouse strains were established from two independent homologous recombinant ES lines. No difference was observed between them in regard to the phenotypes described below. As controls, previously established *Otx2* null mutants (Matsuo et al., 1995) were examined in the same generations by the same crosses. The F_2 heterozygous and F_3 homozygous phenotypes of the null mutation were identical to those previously reported. The mutated loci are designated below as $Otx2^{+/-}$ and $Otx2^{-/-}$ for the respective heterozygous and homozygous null mutation and $Otx2^{+/otx1}$ and $Otx2^{otx1/otx1}$ for the respective heterozygous and homozygous knock-in mutations.

To determine whether the knocked-in *Otx1* gene is properly expressed in place of the *Otx2* gene, we first examined β-Gal expression in mice in which the *Otx2* gene was replaced with the *lacZ* gene using the same strategy. The mice showed β-Gal expression in the visceral endoderm, anterior mesendoderm, the anterior neuroectoderm to the caudal boundary at the midbrain/hindbrain junction, cephalic mesenchyme and other regions known to express *Otx2*, by in situ hybridization (Fig. 6P; Acampora et al., 1995). Second, we determined the mRNA level in heterozygous ($Otx2^{+/otx1}$) and homozygous knock-in ($Otx2^{otx1/otx1}$) embryos by quantitative RT-PCR at 7.5 dpc when normally *Otx2*, but not *Otx1*, is expressed (Fig. 2A, lane 2). In the $Otx2^{otx1/otx1}$ embryos, *Otx1* was expressed instead of

Otx2 as predicted (lane 3). In addition, analysis of the *Otx2*$^{+/otx1}$ embryos showed that *Otx1* and *Otx2* were expressed at approximately the same levels (lane 4). Third, we conducted in situ hybridization analyses of *Otx1* and *Otx2* expressions in *Otx2*$^{+/otx1}$ embryos at the same stage. The *Otx1*-positive region was almost identical in pattern to the *Otx2*-positive region (Fig. 2B). In situ analysis of *Otx2*$^{otx1/otx1}$ embryos also verified the replacement of *Otx2* expression by *Otx1* in knock-in embryos (Fig. 6B). Finally, we analyzed the *Otx1* protein product in 8.0 dpc knock-in embryos, the stage at which *Otx2* is expressed mainly in the anterior neuroectoderm of wild-type embryos; at this stage *Otx2* expression in the visceral endoderm and definitive mesendoderm is residual, and *Otx1* expression in the neuroectoderm is minimal (Fig. 6P-R, Fig. 9). The knocked-in *Otx1* was expressed as a 40 kDa protein product as predicted (Fig. 2C).

Otx2 heterozygous phenotype

Otx2 heterozygous mutants (*Otx2*$^{+/-}$) exhibit craniofacial malformations (Matsuo et al., 1995), a phenotype observed in a C57BL/6 genetic background but suppressed in a CBA background. TT2 ES cells have the F_1 genetic background of CBA and C57BL/6 mice (Yagi et al., 1993a). Thus, it was necessary to determine which *Otx2* allele, C57BL/6 or CBA, was targeted in the knock-in and the null mutations. To do so we found a polymorphic site located about 1.5 kb upstream of the translation start site that discriminates between the CBA and C57BL/6 *Otx2* loci (Oyanagi et al., 1997). The targeting vectors for the null and knock-in mutations were both made using CBA *Otx2* genomic DNA. An example of the analysis is shown in Fig. 3A. When the F2 *Otx2*$^{+/-}$ or *Otx2*$^{+/otx1}$ mice were mated with C57BL/6 mice, all the wild-type offspring bore only the C57BL/6-derived *Otx2* allele, whereas all the heterozygotes bore both C57BL/6- and CBA-derived alleles. In turn, when the F2 *Otx2*$^{+/-}$ or *Otx2*$^{+/otx1}$ mice were mated with CBA mice, all the wild-type offspring bore both C57BL/6- and CBA-derived alleles, while all the heterozygotes bore only the CBA-derived *Otx2* allele. This data confirms that in both the null and the knock-in mutations, recombination took place in the CBA *Otx2* allele of TT2 ES cells. Thus, any potential difference between the heterozygous phenotype observed in the null mutation and the knock-in mutation is not attributable to a difference in the origin of targeted *Otx2* allele.

The *Otx2*$^{+/-}$ phenotype in a C57BL/6 genetic background is variable (Matsuo et al., 1995). Acephaly was observed in about 3% of the *Otx2*$^{+/-}$ mice but never seen in *Otx2*$^{+/otx1}$ mice (Fig. 3B,C). About 53% of the *Otx2*$^{+/-}$ mice had defects in the eyes and lower jaw, but similar

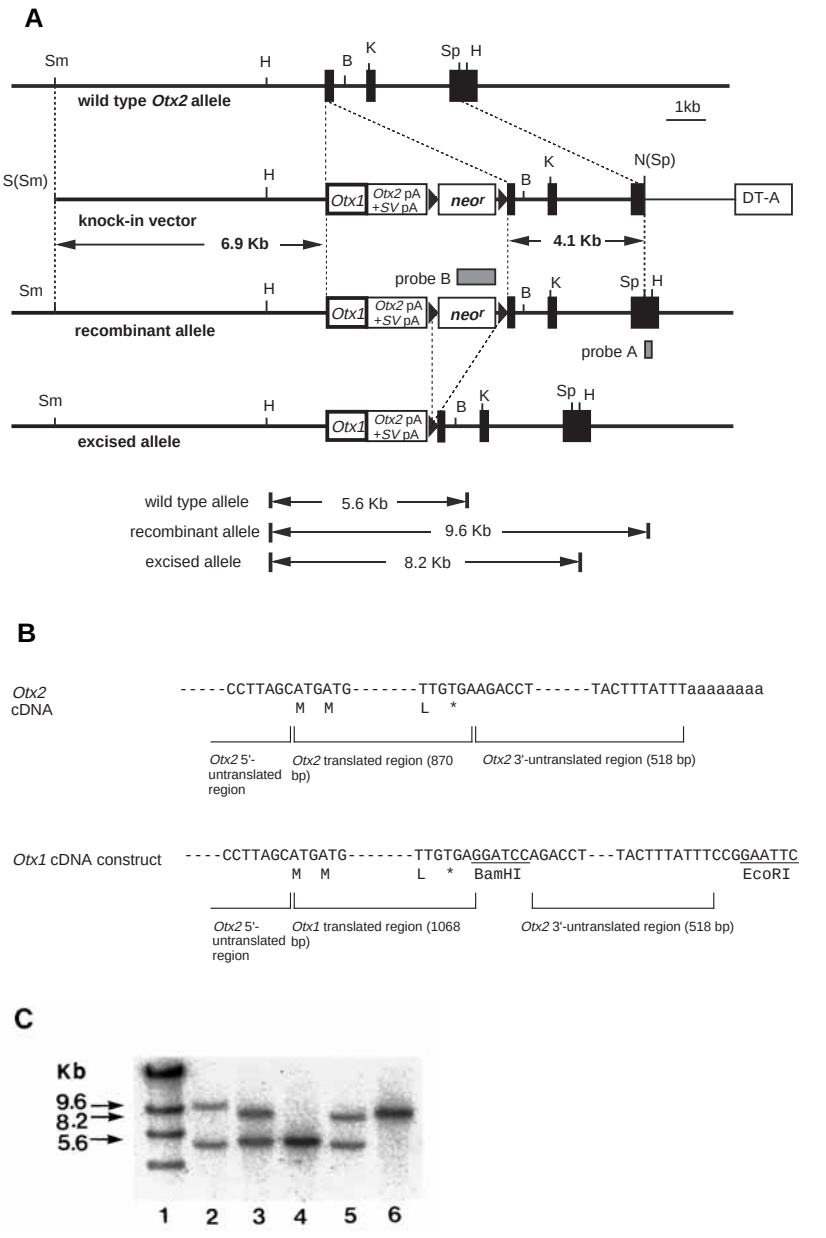


Fig. 1. The *Otx1* knock-in mutation in the *Otx2* locus. (A) Diagrammatic representations of the wild-type *Otx2* allele, knock-in vector, recombinant allele and excised allele. In the targeting construct, *Otx1* cDNA (see B) with polyadenylation signals from *Otx2* and SV40 early genes and the neomycin resistance gene driven by the *PGK1* promoter and without a polyadenylation signal (*neo*^{r}) were inserted into the translational start site of the *Otx2* gene; *neo*^{r} was flanked with loxP. The length of the homologous region was 6.9 kb and 4.1 kb at 5′ and 3′ ends of the insert, respectively. DT-A indicates the diphtheria toxin A gene fragment driven by the MC1 promoter for negative selection (Yagi et al., 1993b). The thick lines indicate *Otx2* genomic sequences; filled boxes, coding regions; a thin line, sequences derived from pBluescript; and triangles, loxP sequences. The locations of probes A and B used for Southern blot analyses are shown. The expected sizes of hybridizing fragments with probe A after the *Hin*dIII digestion are indicated at the bottom. Abbreviations: B, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; N, *Not*I; S, *Sal*I; Sp, *Sph*I; Sm, *Sma*I. (B) The *Otx1* cDNA construct. All noncoding sequences are replaced with those of *Otx2* cDNA. Stars indicate the stop codon. (C) An example of Southern blot analysis after digestion with *Hin*dIII and hybridization with probe A. Lanes: 1, size markers; 2, a homologous recombinant ES clone; 3, an F_1 male in which *neo*^{r} was excised with Cre recombinase; 4, an F_3 wild-type embryo; 5, an F_3 heterozygote; 6, an F_3 homozygous knock-in mutant.

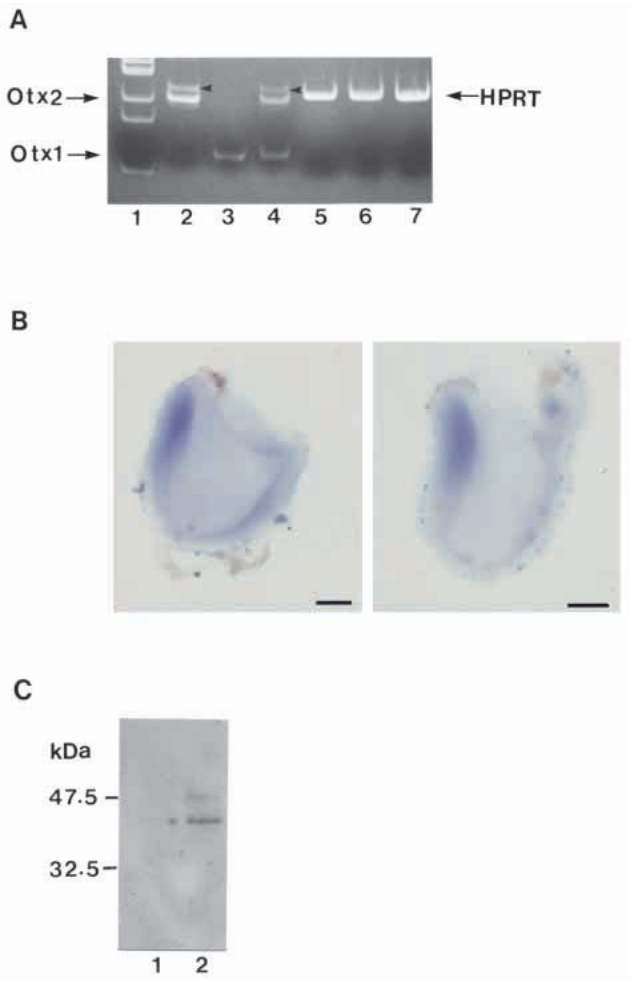


Fig. 2. Characterization of the *Otx1* knock-in mutation in the *Otx2* locus. (A) RT-PCR analysis of *Otx1* and *Otx2* expression at 7.5 dpc. Lanes: 1, size markers; 2 and 5, wild type; 3 and 6, $Otx2^{otx1/otx1}$; 4 and 7, $Otx2^{+/otx1}$. RT-PCR was performed for both *Otx1* and *Otx2* expression in each sample (Lanes 2-4); HPRT expression is shown as a control (Lanes 5-7). Note the absence of *Otx1* transcripts in the wild-type embryo at this stage (Lane 2), the absence of *Otx2* transcripts in the $Otx2^{otx1/otx1}$ embryo (Lane 3) and nearly equal amounts of *Otx1* and *Otx2* transcripts in the $Otx2^{+/otx1}$ embryo (Lane 4). Under the conditions used for RT-PCR of *Otx2* an extra band of unknown origin is apparent (arrowheads). (B) In situ hybridization of *Otx2* (left) and *Otx1* (right) in 7.5 dpc $Otx2^{+/otx1}$ embryos. Scale bars, 100 μm. (C) Western blot analysis of *Otx1* expression in 8.0 dpc wild-type (lane 1) and $Otx2^{otx1/otx1}$ (lane 2) embryos. No Otx1 protein was detected in wild-type embryos at this stage, and the antibody does not cross-react with Otx2 protein. Multiple bands may be due to posttranslational modification of Otx1 protein, which has been reported for Otx2 protein (Mallamaci et al., 1996); the consequences and nature of such modification are not known. The positions of molecular size markers are indicated on the left.

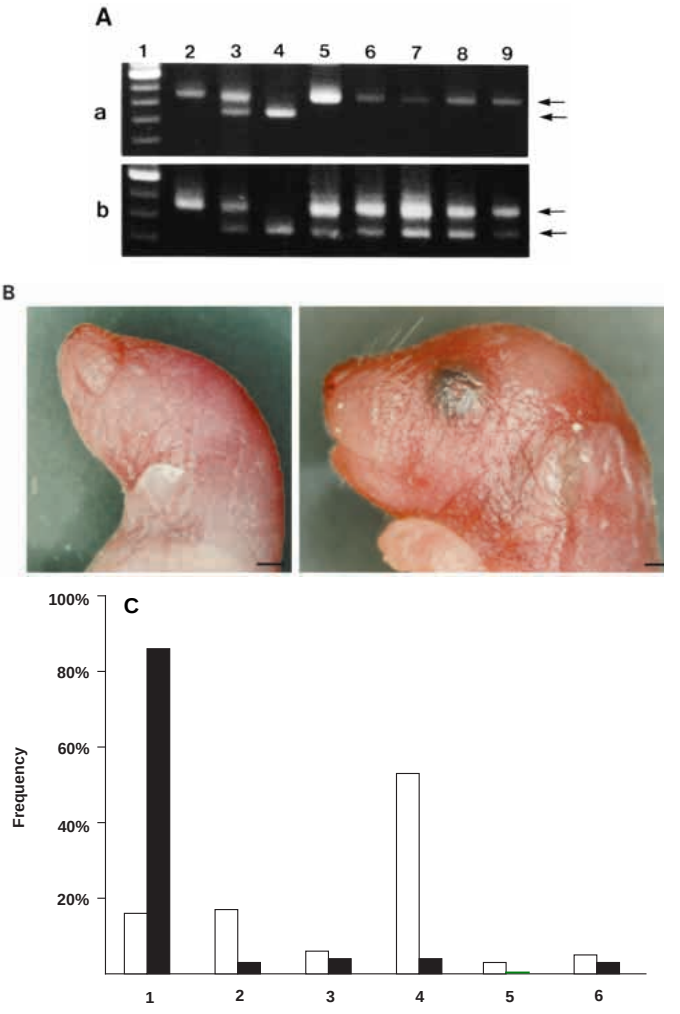


Fig. 3. Rescue of otocephalic phenotype in *Otx2* heterozygous mutants by *Otx1*. (A) An example of assignment of the targeted allele. An F_2 $Otx2^{+/otx1}$ male was mated with a C57BL/6 female, and their offspring were genotyped for both the *Otx1* knock-in mutation into *Otx2* locus and the presence of C57BL/6 and CBA *Otx2* allele (Oyanagi et al., 1997). Lanes: 1, size markers; 2, a C57BL/6 mouse; 3, TT2 cells; 4, a CBA mouse; 5-9, wild-type (a) or mutant (b) offspring. (B) Lateral views of typical $Otx2^{+/-}$ (left) and $Otx2^{+/otx1}$ (right) newborn mice. 53% of $Otx2^{+/-}$ mutants had defects in both eyes and lower jaw, whereas 86% of $Otx2^{+/otx1}$ mutants were apparently normal. Scale bars, 1 mm. (C) Frequency of cranial defects in $Otx2^{+/-}$ (white columns) and $Otx2^{+/otx1}$ (black columns) newborn mice. The frequency is given for 156 $Otx2^{+/-}$ and 215 $Otx2^{+/otx1}$ mutants examined; total is 100%. Columns: 1, mice with no apparent defects; 2, defects in eyes; 3, in jaw; 4, in both eyes and jaw; 5, no head; 6, other defects such as ethmocephaly and short nose (Matsuo et al., 1995).

defects were seen in only 3% of the $Otx2^{+/otx1}$ mice. The proportion of $Otx2^{+/-}$ mice showing defects only in eyes or only in lower jaw was approximately 17% and 6%, respectively; in $Otx2^{+/otx1}$ mice the percentages were 3% and 4%, respectively. In the $Otx2^{+/-}$ mice, only 16% showed no apparent defects, yet 86% of $Otx2^{+/otx1}$ mice were apparently normal. Craniofacial defects seen in $Otx2^{+/-}$ mice (i.e., in the trabecular components of cranium, the opthalmic branch of the trigeminal nerve and in mesencephalic trigeminal neurons (Matsuo et al., 1995)) were also largely restored in $Otx2^{+/otx1}$ mice (data not shown). We previously identified the craniofacial anomalies of $Otx2^{+/-}$ mice as defects in cephalic neural crest cells (Matsuo et al., 1995; Kimura et al., 1997). Therefore we suggest that *Otx1* can substitute for *Otx2* function in these cells.

Homozygous knock-in phenotype

Otx2$^{-/-}$ embryos did not develop beyond 9.5 dpc (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996), but *Otx2*$^{otx1/otx1}$ mice were live-born, although they did not survive. Before birth, at 18.5 dpc, 23 out of 56 offspring from crosses among heterozygotes were homozygous knock-in mutants. These *Otx2*$^{otx1/otx1}$ embryos, however, suffered from severe otocephaly. Some embryos lacked the rostral head, while most developed some rostral structures (Fig. 4A-D). Otic vesicles, the medulla oblongata, the trigeminal ganglion, choroid plexus in the fourth cerebral ventricle, the trachea, the entrance to the esophagus, the hyoid bone, Meckel's cartilage and the basisphenoid bone were present in all prenatal knock-in embryos. Many *Otx2*$^{otx1/otx1}$ embryos developed a tongue, oropharynx and mandible (though small) with teeth and lower lip; the maxillary bone was never observed. The cerebellum, pons and some structures anterior to both were also formed in some embryos, but the forebrain, hypophysis and most anterior structures such as eyes or olfactory bulbs were never formed. Thus, knocked-in *Otx1* can rescue some but not all *Otx2* functions in the rostral head.

Otx2$^{otx1/otx1}$ embryos were next examined at 11.5 dpc (Fig. 4E,F). Otic vesicles and the trigeminal ganglion were present. The isthmic constriction was obscure in some embryos but observable in most, and more caudal structures were apparently normal. An infundibulum-like structure was present, as was the cephalic flexure, and some anterior structures were observed. Eyes were never formed.

Early defects in the rostral brain

To determine the affected regions more precisely, an analysis was undertaken with molecular markers at 8.5 dpc. Development was generally retarded in homozygous knock-in embryos. The *Otx2*$^{-/-}$ embryos never develop regions anterior to r3 (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). In homozygous knock-in embryos, *Krox20* expression in r3 and r5 and *Fgf8* expression in the isthmus were normal (Fig. 5A-D); *Pax2* expression in the isthmus was present though weak compared to wild type. *Fgf8* was normally expressed in a transverse band in the 9.5 dpc isthmus (data not shown). In wild-type embryos *Otx1* and *Otx2* are expressed in forebrain and midbrain with the caudal boundary of both at the junction with the isthmus. *Otx2*$^{otx1/otx1}$ embryos had *Otx1*-positive region, although the region was greatly reduced (Fig. 5G,H). No *Otx2*

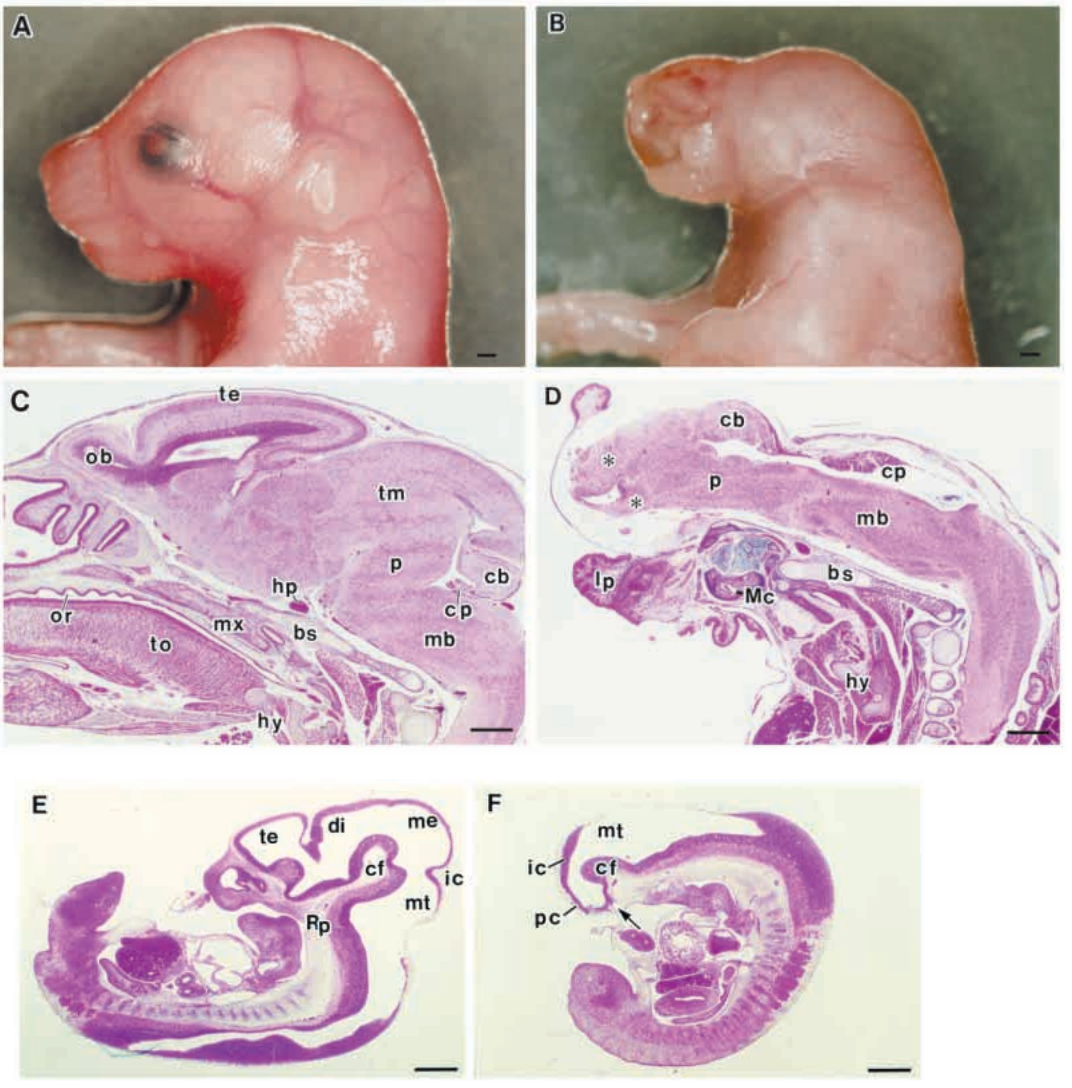


Fig. 4. Morphological analysis of *Otx2*$^{otx1/otx1}$ embryos. (A,C,E) Wild-type and (B,D,F) *Otx2*$^{otx1/otx1}$; (A-D) 18.5 dpc and (E,F) 11.5 dpc embryos. Structures marked by stars anterior to the cerebellum (cb) and pons (p) in D were not identified. In 11.5 dpc *Otx2*$^{otx1/otx1}$ embryos, the isthmic constriction (ic) and posterior commissure (pc) were barely detectable, and some structures developed anteriorly. An arrow indicates the infundibulum-like structure. Though not shown, otic vesicles and trigeminal ganglia were present. Abbreviations: bs, basisphenoid bone; cf, cephalic flexure; cp, choroid plexus; di, diencephalon; hp, hypophysis; hy, hyoid bone; lp, lower lip; mb, medulla oblongata; me, mesencephalon; Mc, Meckel's cartilage; mt, metencephalon; mx, maxilla; ob, olfactory bulb; or; oropharynx; Rp, Rathke's pouch; te, telencephalon; tm, tegmentum; to, tongue. Scale bars, 500 μm.

expression was detected as expected (data not shown). In wild-type embryos *En1* is expressed from r1 to the midbrain, and *Wnt1* is expressed from the isthmus to the caudal diencephalon. In the *Otx2*$^{otx1/otx1}$ embryos, both *En1* and *Wnt1* expressions were markedly reduced (Fig. 5I-L). It is noteworthy that almost no morphological structure was observed anterior to the *Wnt1*-positive region (Fig. 5K,L). Normally *Emx2* is expressed in the telencephalon and anterior diencephalon; in homozygous knock-in embryos its expression was barely detectable (Fig. 5A,B). In wild-type embryos *Six3* expression is seen in the anterior ridge of the forebrain. In the *Otx2*$^{otx1/otx1}$ embryos, this expression was absent (Fig. 5M,N), but expression in the ventral diencephalon, which may correspond to the future infundibulum, was retained. *Hesx1* was expressed weakly in the future Rathke's pouch but not seen in the anterior neuroectoderm (Fig. 5O,P). *Fgf8* expression was also observed in the ventral diencephalon but not in the anterior neural ridge (Fig. 5C,D). Overall, marker analysis showed normal development of the isthmus and r1/2, defects in the midbrain and loss of dorsal forebrain. Variation in these markers was minimal in 8.5 dpc *Otx2*$^{otx1/otx1}$ embryos, and alterations in expression of markers compared to wild-type patterns were predictive of the 11.5 dpc phenotype.

Induction of rostral neuroectoderm

Defects in homozygous knock-in embryos were further examined at 7.5-7.75 dpc, the initial stage of rostral neuroectoderm development. *Otx2* (Simeone et al., 1993), *Six3* (Oliver et al., 1995), *Fkh2* (Kaestner et al., 1995), *Hesx1/Rpx* (Thomas and Beddington, 1996; Hermesz et al., 1996) and *Pax2* (Rowitch and McMahon, 1995) are the earliest markers of rostral neuroectoderm. *Otx2*$^{-/-}$ embryos never expressed these markers at this stage (Fig. 6C,F,I,L,O); *Pax2* expression was not observed in our 7.5-8.5 dpc *Otx2*$^{-/-}$ embryos (Fig. 6O) despite the recent observation by Rhinn et al. (1998). In the *Otx2*$^{otx1/otx1}$ embryos, knocked-in *Otx1* expression was indistinguishable from *Otx2* expression seen in wild-type embryos (Fig.

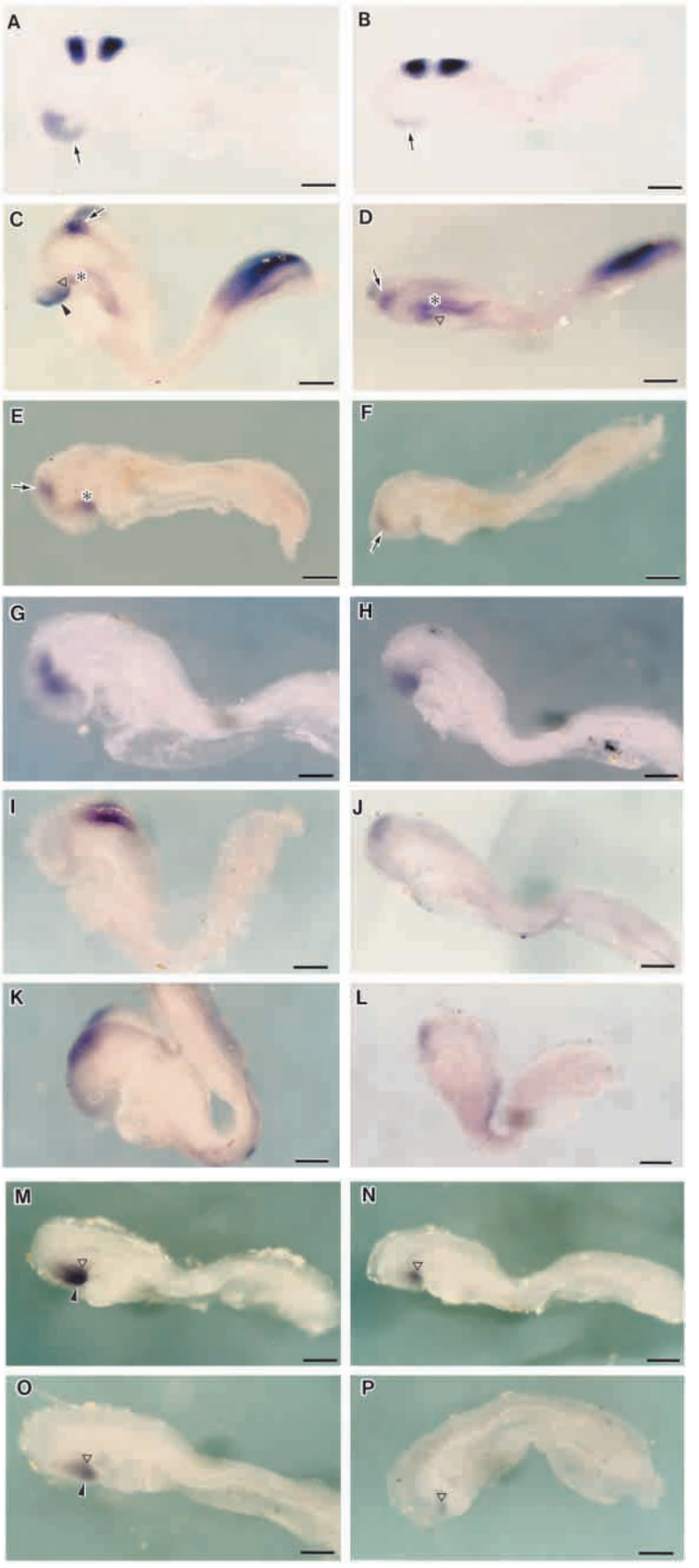


Fig. 5. Expression of anterior neural markers in 8.5 dpc rostral brain. (A,C,E,G,I,K,M,O) Wild-type and (B,D,F,H,J,L,N,P) *Otx2*$^{otx1/otx1}$. (A,B) *Krox20* and *Emx2*, (C,D) *Fgf8*, (E,F) *Pax2*, (G,H) *Otx1*, (I,J) *En1*, (K,L) *Wnt1*, (M,N) *Six3* and (O,P) *Hesx1* expressions. In *Otx2*$^{-/-}$ embryos, the *Krox20*-positive r3 is at the most anterior end, and the other genes are never expressed (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). Arrows in (A,B) show *Emx2* expression in forebrain that is faint in *Otx2*$^{otx1/otx1}$ embryos, and in (C-F) expression in isthmus. Open triangles in (C,D,M,N) indicate expression in ventral diencephalon, which corresponds to the future infundibulum, and in (O,P) Rathke's pouch; arrowheads in (C,M,O) indicate *Fgf8*, *Six3* and *Hesx1* expression in the anterior neural ridge or neuroectoderm that is absent in *Otx2*$^{otx1/otx1}$ embryos (D,N,P). Asterisks in C,D indicate *Fgf8* expression in the foregut, and in E *Pax2* expression in the optic stalk. Scale bars, 150 μm.

6A,B). *Six3*, *Fkh2* and *Pax2* expression patterns were also normal in homozygous knock-in embryos (Fig. 6D,E,G,H). The region anterior to *Pax2* expression was, however, somewhat reduced (Fig. 6M,N), and expression of all these markers decreased by 8.0 dpc (data not shown). *Hesx1* expression was faint even in the 7.5 dpc $Otx2^{otx1/otx1}$ embryos (Fig. 6J,K). We also compared β-Gal expression at 7.75 dpc in $Otx2^{+/lacZ}$ and $Otx2^{otx1/lacZ}$ embryos (Fig. 6P-R). In $Otx2^{+/lacZ}$ embryos at this stage, β-Gal expression was found mainly in anterior neuroectoderm, and expression in anterior definitive mesendoderm was residual. In $Otx2^{otx1/lacZ}$ embryos at this stage β-Gal expression was found in anterior mesendoderm and induced in anterior neuroectoderm, though the level was lower than that seen in $Otx2^{+/lacZ}$ embryos. The *Otx1* dosage effect should be kept in mind when comparing levels of β-Gal expressions (Fig. 6P-R) with those of *Otx2* and *Otx1* mRNA (Fig. 6A,B).

About half of the $Otx2^{-/-}$ embryos (11/20) undergo abnormal gastrulation, exhibiting a characteristic constriction at the junction of the extraembryonic and embryonic ectoderm at early to mid-streak stage (Fig. 7C). Similar abnormalities were also seen in *Lim1* and *HNF3β* mutants (Ang and Rossant, 1994; Weinstein et al., 1994; Shawlot and Behringer, 1995). Such a phenotype was never observed in $Otx2^{otx1/otx1}$ embryos (0/20) (Fig. 7B), which also expressed *Brachyury* normally (Fig. 7D-F). In the $Otx2^{-/-}$ embryos, *HNF3β*-positive anterior mesendoderm was not formed, but $Otx2^{otx1/otx1}$ embryos developed the mesendoderm (Fig. 7G-I). In wild-type embryos, *cer-l* is expressed in anterior mesendoderm to the embryonic/extraembryonic junction (Belo et al., 1997; Biben et al., 1998; Shawlot et al., 1998) (Fig. 7J). In $Otx2^{-/-}$ embryos *cer-l* expression either did not extend to the junction (Fig. 7L) or frequently remained in the distal tip of the mesendoderm (Biben et al., 1998); however, *cer-l* was expressed normally in the anterior mesendoderm of $Otx2^{otx1/otx1}$ embryos (Fig. 7K).

Cooperative function of *Otx2* and *Otx1* in development of mesencephalon and diencephalon

Otx1 expression begins at 8.0 dpc, around the 1-3 somite stage (Simeone et al., 1992; Suda et al., 1997). *Otx2* function in the neuroectoderm after this stage is apparent from the phenotype of *Otx2* and *Otx1* double heterozygous mutants ($Otx1^{+/-}$ $Otx2^{+/-}$): these mice show marked defects in mesencephalon and caudal diencephalon, while defects are not seen in either $Otx1^{-/-}$ or $Otx2^{+/-}$ mutants (Suda et al., 1996, 1997). The defects observed in the double heterozygotes take place at the time of brain regionalization occurring around the 3 to 6 somite stage. Functional equivalency between *Otx1* and *Otx2* at this stage cannot be deduced from the $Otx2^{otx1/otx1}$ phenotype, since

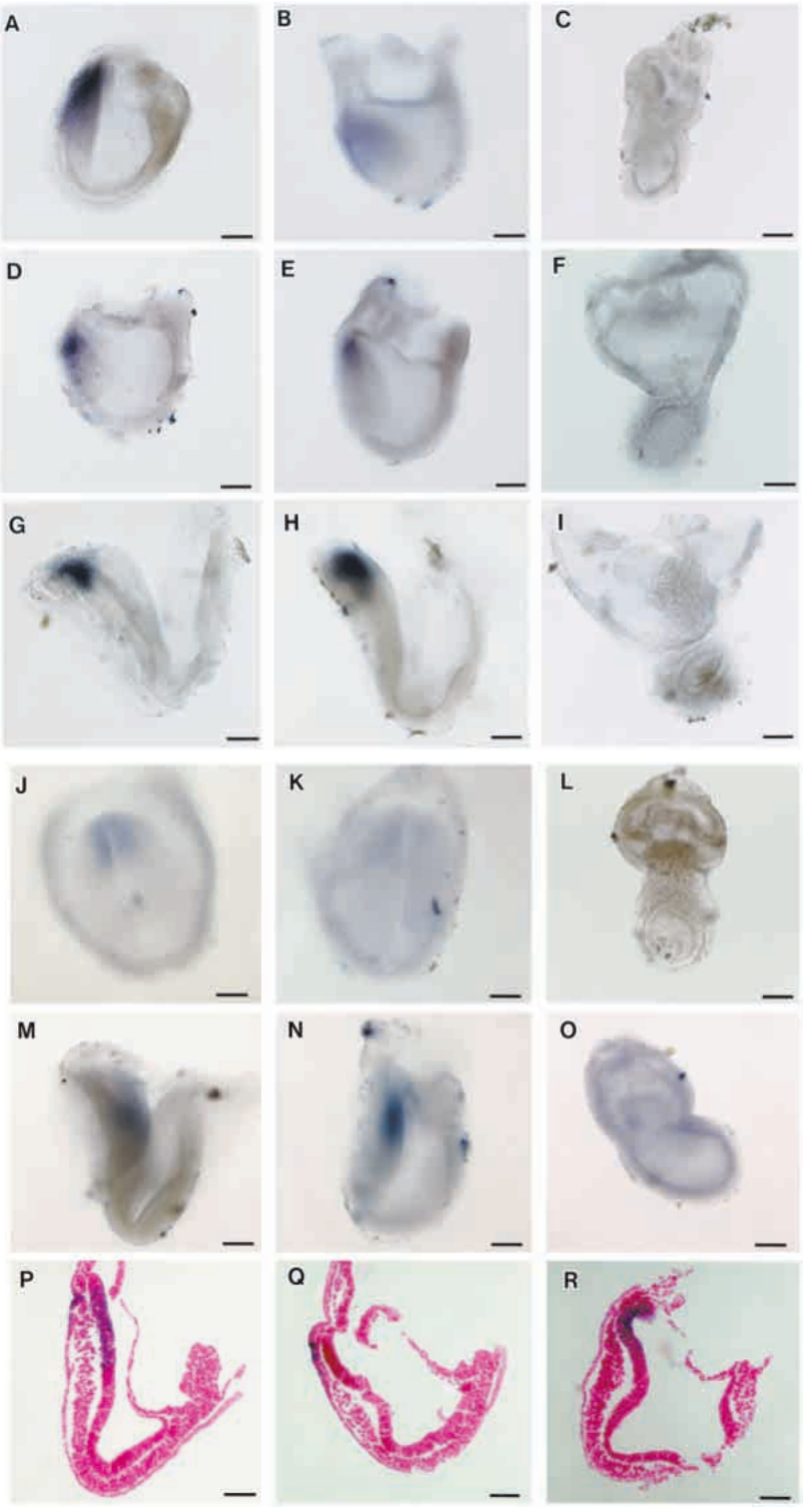


Fig. 6. Expression of anterior neural markers at 7.5-7.75 dpc. (A,D,G,J,M) Wild type, (B,E,H,K,N) $Otx2^{otx1/otx1}$, (C,F,I,L,O) $Otx2^{-/-}$, (P) $Otx2^{+/lacZ}$ and (Q,R) $Otx2^{otx1/lacZ}$. (A,C) *Otx2*, (B) *Otx1*, (D-F) *Six3*, (G-I) *Fkh2*, (J-L) *Hesx1*, (M-O) *Pax2* and (P-R) β-Gal expressions. J-L show ventral views and others are lateral views. In $Otx2^{otx1/otx1}$ embryos, *Otx2* expression was not seen (Fig. 2A, lane 3), nor is endogenous *Otx1* expressed at this stage (Fig. 2A, lane 2). Thus *Otx1* expression in the $Otx2^{otx1/otx1}$ embryo (B) represents that of the knocked-in *Otx1*. The embryo shown in Q in which β-Gal is expressed in anterior mesendoderm is less advanced than the embryo in R where β-Gal expression is already initiated in anterior neuroectoderm. Scale bars, 100 μm.

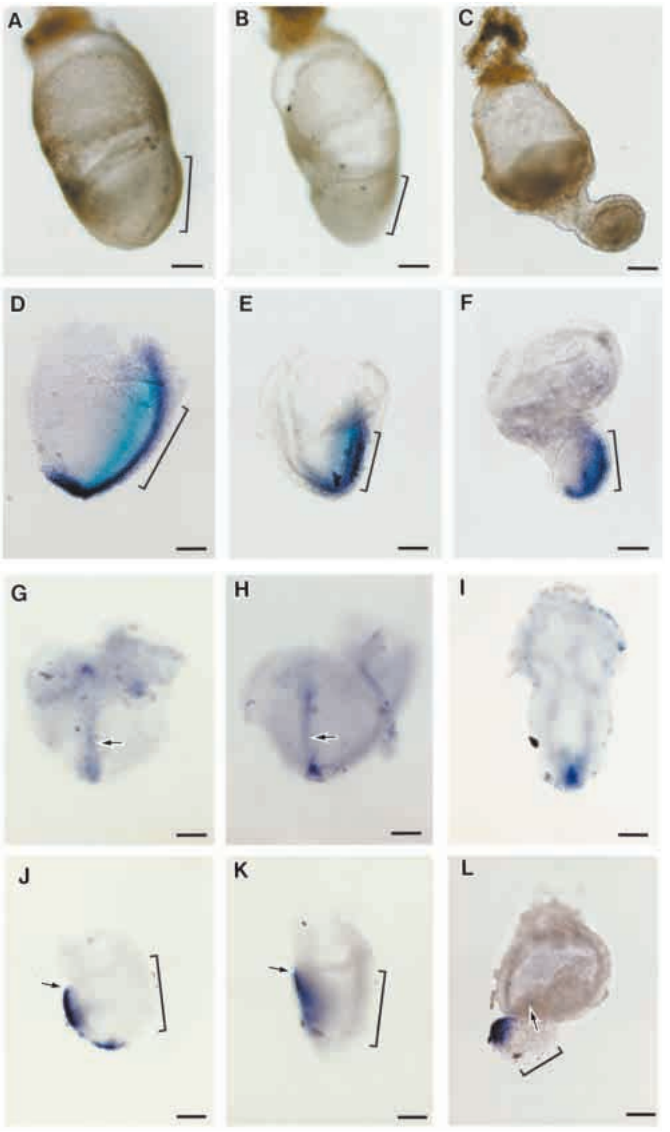


Fig. 7. Rescue of 7.5 dpc $Otx2^{-/-}$ defects in $Otx2^{otx1/otx1}$ mutants. (A,D,G,J) Wild type, (B,E,H,K) $Otx2^{otx1/otx1}$ and (C,F,I,L) $Otx2^{-/-}$. (A-C) Morphological features, and expression of (D-F) *Brachyury*, (G-I) *HNF3β* and (J-L) *cer-l*. G-I show ventral views and the others show lateral views. Abnormal gastrulation seen in $Otx2^{-/-}$ embryos (C) is never observed in $Otx2^{otx1/otx1}$ embryos (B). $Otx2^{-/-}$ embryos never develop *HNF3β*-positive anterior mesendoderm (I), but the mesendoderm (arrows in G,H) is formed in $Otx2^{otx1/otx1}$ embryos. In $Otx2^{-/-}$ embryos *cer-l* expression does not extend to the embryonic/extraembryonic junction (L), but is always normal in the anterior mesendoderm of $Otx2^{otx1/otx1}$ embryos (K). Arrows in J-L indicate the junction. Brackets indicate the primitive streak. Scale bars, 100 μm.

defects were seen in the anterior neuroectoderm of $Otx2^{otx1/otx1}$ mutants before the onset of *Otx1* expression. Therefore to examine whether *Otx1* can substitute for *Otx2* function at this stage $Otx1^{+/-}Otx2^{+/otx1}$ double heterozygotes were analyzed.

In 10.5 dpc $Otx1^{+/-}Otx2^{+/-}$ embryos, the region between the otic vesicles and the isthmic constriction was enlarged (Fig. 8A,B), whereas that between the constriction and the sulcus telodiencephalicus was shortened (Fig. 8A-D), as we previously reported (Suda et al., 1998): the telencephalon was smaller, the invagination of the sulcus telodiencephalicus was poor and the isthmic constriction was not distinct. In the majority of 10.5 dpc $Otx1^{+/-}Otx2^{+/otx1}$ embryos, the mesencephalon was somewhat smaller, the isthmic constriction was somewhat extended, and r1 was slightly expanded. However, the sulcus telodiencephalicus was present in a normal manner, and no defects were apparent in telencephalon or diencephalon. In prenatal $Otx1^{+/-}Otx2^{+/-}$ mutants, the midbrain, pretectum and dorsal thalamus were vestigial, while the anterior hindbrain, cerebellum and pons were expanded. The ventral thalamus, cerebral hemispheres and the hippocampal region were mildly reduced in size. In contrast, these defects were almost completely restored in most of the $Otx1^{+/-}Otx2^{+/otx1}$ mutants, although in some mutants the restoration was partial. Thus *Otx2* function in the neuroectoderm after the onset of *Otx1* expression may essentially be rescued by *Otx1*.

DISCUSSION

We have used a knock-in strategy to examine the functional equivalency of *Otx1* and *Otx2* genes. Our findings suggest that *Otx1* can substitute for *Otx2* function in the visceral endoderm, the anterior mesendoderm and cephalic neural crest cells. *Otx1* can also replace *Otx2* function in the anterior neuroectoderm after the onset of *Otx1* expression. *Otx1* cannot, however, substitute for *Otx2* function in the neuroectoderm prior to the onset of *Otx1* expression. The amino acid identity between mouse *Otx1* and *Otx2* is 98% in homeodomain, 81% in the N-terminal region and 44% in the C-terminal region (Simeone et al., 1993). A chronology of rostral brain development and mutant defects described in this paper are shown schematically in Fig. 9, along with spatiotemporal changes in *Otx* gene expression. *Otx1* and *Otx2* functions in later neurogenesis fall outside the scope of this study.

Knock-in mutation

$Otx2^{+/-}$ and $Otx1^{+/-}Otx2^{+/-}$ defects were restored in a majority of $Otx2^{+/otx1}$ and $Otx1^{+/-}Otx2^{+/otx1}$ mutants, respectively, but restoration was partial in some embryos. The prenatal $Otx2^{otx1/otx1}$ phenotype was also variable with possible secondary defects, although it was fairly consistent at earlier stages. Whether a knocked-in gene is expressed as precisely as the replaced gene is a critical question, and that issue is particularly relevant to defects seen in early anterior neuroectoderm, which were not rescued in $Otx2^{otx1/otx1}$ embryos. Our belief that the knocked-in *Otx1* gene is expressed properly in the neuroectoderm is based on, (1) the β-Gal expression pattern seen using the same knock-in strategy, (2) RT-PCR detection of *Otx1* mRNA expression at 7.5 dpc when *Otx1* is not normally expressed, and (3) in situ analysis at the same stage.

In $Otx2^{+/lacZ}$ embryos, however, β-Gal was not expressed in the epiblast as reported by Acampora et al. (1995). This finding suggests that posttranscriptional and/or posttranslational regulatory elements required for expression in the epiblast are missing in the *lacZ* knock-in construct, and that such regulation might be also required for expression of knocked-in *Otx1* in early anterior neuroectoderm. We, however, consider this possibility unlikely for several reasons. First, all noncoding sequences of the knocked-in *Otx1* cDNA construct were replaced with corresponding sequences of *Otx2*. Second, in

Otx2$^{+/lacZ}$ and *Otx2*$^{otx1/lacZ}$ embryos in which the *lacZ* gene was knocked-in, β-Gal was expressed in anterior neuroectoderm. Third, the rescue of *Otx1*$^{+/-}$*Otx2*$^{+/-}$ defects in *Otx1*$^{+/-}$*Otx2*$^{+/otx1}$ embryos indicates that knocked-in *Otx1* is expressed in the neuroectoderm. Finally, western blot analysis of Otx1 protein in 8.0 dpc *Otx2*$^{otx1/otx1}$ embryos suggests that the protein is produced in the anterior neuroectoderm, because at this stage in wild-type embryos, *Otx2* is expressed mainly in anterior neuroectoderm and *Otx1* is not expressed.

Early *Otx2* functions

Otx2 mRNA is first expressed in the mouse epiblast (Simeone et al., 1993). A role in cell proliferation has been suggested (Ang et al., 1996), but *Otx2* function is not yet determined. Prior to primitive streak formation *Otx2* is also expressed in the visceral endoderm (Acampora et al., 1995). That expression localizes to the future anterior region of the embryo, as does expression of *cer-l*, *Hesx1*, *Lim1*, *nodal*, *Hex* and *HNF3β* (Hermesz et al., 1996; Thomas and Beddington, 1996; Belo et al., 1997; Varlet et al., 1997; Beddington and Robertson, 1998; Biben et al., 1998; Shawlot et al., 1998; Thomas et al., 1998). Expression of these genes in the extraembryonic endoderm may play a role in head organizer activity, the loss of which leads to developmental failure of structures anterior to r3 in *Otx2*- and *Lim1*-deficient embryos (Beddington and Robertson, 1998; Rhinn et al., 1998). *Otx2*, *Lim1* and *HNF3β* mutants all exhibit abnormal gastrulation (Ang and Rossant, 1994; Weinstein et al., 1994; Shawlot and Behringer, 1995). As primitive streak formation progresses, *Otx2* expression in the epiblast is lost caudally and becomes confined to the anterior region through the three germ layers (Simeone et al., 1993; Acampora et al., 1995). In avians, *Otx2* mRNA expression in the epiblast once disappears, becomes confined to Hensen's node and then localizes to the anterior neuroectoderm (Bally-Cuif et al., 1995).

In *Otx2*$^{otx1/otx1}$ embryos, gastrulation defects were not observed, and *HNF3β*-positive anterior mesendoderm was formed. These embryos expressed *cer-l*, a candidate rostral neuroectoderm inducer (Belo et al., 1997; Biben et al., 1998; Shawlot et al., 1998), normally. Expression of *Six3*, *Fkh2* and *Pax2*, and knocked-in *Otx1* and β-Gal suggest induction of anterior neuroectoderm in knock-in embryos. Thus we conclude that any early functions of *Otx2* in the epiblast, in visceral endoderm and in anterior mesendoderm are substitutable with *Otx1*.

Otx2 function in establishment of rostral neuroectoderm

Morphologically, rostral brain develops as prosencephalic and mesencephalic vesicles (Puelles et al., 1987). The caudal limit of *Otx2* expression in anterior neuroectoderm, though initially obscure, becomes distinct in the mesencephalic vesicle; the *Otx2*-positive region generates the midbrain and the *Otx2*-negative region becomes the isthmus (Bally-Cuif and Wassef, 1995; Millet et al., 1996). *Otx1* expression occurs around the time of this process at 8.0 dpc or the 1-3 somite stage (Simeone et al., 1993; Suda et al., 1997). Thus, the establishment of anterior neuroectoderm is initiated in the absence of the *Otx1* expression.

Analysis using molecular markers at 7.5 to 8.5 dpc suggested that anterior neuroectoderm was induced but failed to develop in *Otx2*$^{otx1/otx1}$ embryos. At 7.5 dpc *Six3*, *Fkh2*, *Pax2* and knocked-in *Otx1* were expressed fairly normally in anterior neuroectoderm, but their expression decreased by 8.0-

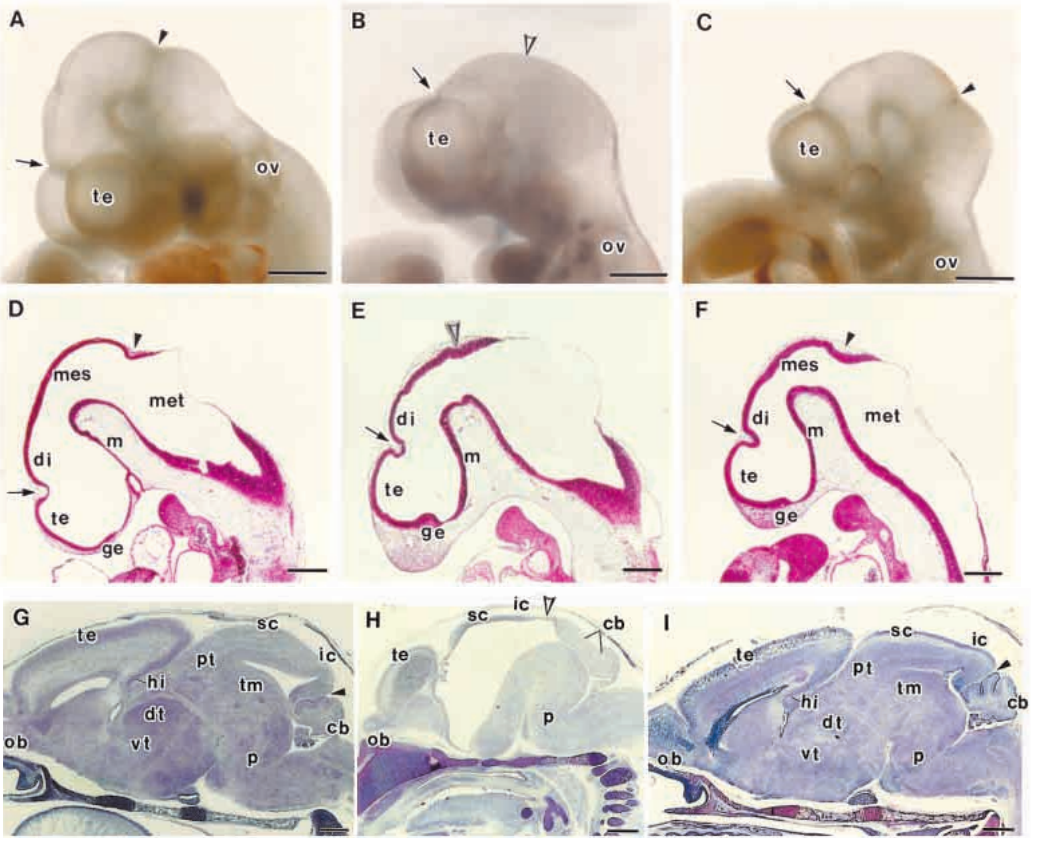
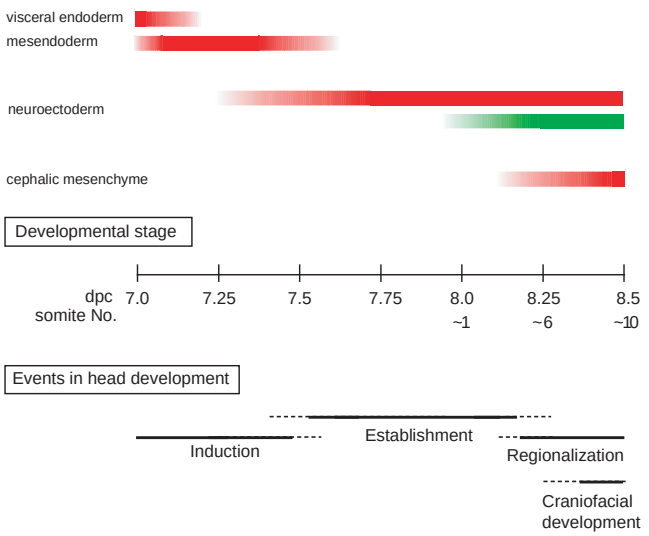


Fig. 8. Rescue of *Otx1*$^{+/-}$*Otx2*$^{+/-}$ defects in *Otx1*$^{+/-}$*Otx2*$^{+/otx1}$ mutants. (A,D,G) Wild type, (B,E,H) *Otx1*$^{+/-}$*Otx2*$^{+/-}$ and (C, F, I) *Otx1*$^{+/-}$*Otx2*$^{+/otx1}$. (A-F) 10.5 dpc, (G,I) newborn and (H) 18.5 dpc. *Otx1*$^{+/-}$ *Otx2*$^{+/-}$ mutants are not live-born (Suda et al., 1996, 1997). Arrows indicate sulcus telodiencephalicus, arrowheads isthmic constriction and open arrowheads lesser isthmic constriction in *Otx1*$^{+/-}$*Otx2*$^{+/-}$ mutants. Abbreviations: cb, cerebellum; di, diencephalon; dt, dorsal thalamus; ge, ganglionic eminence; hi, hippocampal region; ic, inferior colliculus; m, mammillary region; me, mesencephalon; mt, metencephalon; ob, olfactory bulb; ov, otic vesicle; p, pons; pt, pretectum; sc, superior colliculus; te, telencephalon; tm, tegmentum; vt, ventral thalamus. Scale bars, 500 μm.



Mutant phenotype

genotype \ event	induction	establishment	regionalization	craniofacial development
Otx2$^{+/-}$	○	○	○	×
Otx2$^{+/otx1}$	○	○	○	○
Otx2$^{-/-}$	×			
Otx2$^{otx1/otx1}$	○	×		
Ep$^{-/-}$ *Ve*$^{+/+}$	○	×		
Otx1$^{-/-}$	○	○	○	○
Otx1$^{+/-}$ *Otx2*$^{+/-}$	○	○	×	
Otx1$^{+/-}$ *Otx2*$^{+/otx1}$	○	○	○	○

Fig. 9. Schematic representation of spatiotemporal changes in *Otx* expression, events in head development and mutant phenotypes. *Otx2* expression shown in red is based on both mRNA in situ and β-Gal analyses (Simeone et al., 1992, 1993; Ang et al., 1994; Acampora et al., 1995; Matsuo et al., 1995; Suda et al., 1997; Kimura et al., 1997; this study); *Otx1* expression shown in green is based on in situ analysis (Simeone et al., 1993; Suda et al., 1997; this study).

8.5 dpc. At 7.5 dpc *Hesx1* expression was barely detectable, and the region anterior to the *Pax2*-positive domain was somewhat reduced in size. Signals from the visceral endoderm and/or anterior mesendoderm may induce *Otx2*, *Six3*, *Fkh2*, *Hesx1* and *Pax2* expression independently in the anterior neuroectoderm; candidate signaling molecules are Cer-l (Belo et al., 1997; Biben et al., 1998; Shawlot et al., 1998), Nodal (Varlet et al., 1997), and Dickkopf-1 (Glinka et al., 1998), all of which may antagonize BMP and Wnt signalling (Glinka et al., 1997; Hsu et al., 1998). The former signalling events may induce *Otx2* expression in the neuroectoderm, which would then function to maintain expression of *Six3*, *Fkh2*, *Hesx1* and *Pax2* in the rostral territory. Timely, albeit weak, expression of *En1* and *Wnt1* at 8.5 dpc in the *Otx2*$^{otx1/otx1}$ neuroectoderm also suggests independent induction of these genes but maintenance by *Otx2*. In *Drosophila* brain *en* and *wg* are targets of *otd* (Cohen and Jurgens, 1991). The knock-in mutation described here suggests that *Otx2* function in the early neuroectoderm prior to the onset of *Otx1* expression cannot be replaced by *Otx1*.

Otx2$^{otx1/otx1}$ defects in early neuroectoderm are similar to those seen in chimeras in which the visceral endoderm of *Otx2*$^{-/-}$ embryos was replaced with wild-type tissue (Ep$^{-/-}$ Ve$^{+/+}$ chimera) (Rhinn et al., 1998). In these chimeras, the prechordal plate was formed and anterior neuroectoderm was induced, but the rostral brain was subsequently deleted. *Six3* and *Pax2* were induced in neuroectoderm at the 0-4 somite stage, but their expression was almost abolished by the 6 to 8 somite stage. *Hesx1* expression was not seen in Ep$^{-/-}$Ve$^{+/+}$ chimeras. These findings support our observations: loss of the *Otx2* function in the visceral endoderm is rescued in *Otx2*$^{otx1/otx1}$ embryos, while loss of *Otx2* in the anterior neuroectoderm is not. In *Otx2*$^{otx1/otx1}$ embryos anterior neuroectoderm is induced but not maintained.

Otx2 function in regionalization of rostral brain

Puelles et al. postulate that there are six prosomers anterior to the midbrain: p1-p6 (Puelles and Rubenstein, 1993; Rubenstein et al., 1994). Recent observations suggest that regionalization in the brain takes place first in three compartments: the region anterior to zona limitans, which generates p3-p6 prosomers; the region between the zona limitans and the isthmus, which generates p1-p2 and the midbrain; and the rhombomeric region posterior to the isthmus. Two organizing centers are postulated at the anterior neural ridge and at the isthmus (Marin and Puelles, 1994; Crossley and Martin, 1995; Crossley et al., 1996; Joyner et al., 1996; Shimamura and Rubenstein, 1997; Houart et al., 1998). The zona limitans, which expresses the signaling molecule SHH (Echelard et al., 1993) and divides the forebrain into two regions, one competent to respond to signals from the isthmus or Fgf8 (p1, p2) and the other not (p3-p6) (Meinhardt, 1983; Martinetz et al., 1991; Figdor and Stern, 1993; Marin and Puelles, 1994; Rubenstein et al., 1994; Bally-Cuiff and Wassef, 1995; Crossley et al., 1996), might be another organizing center. It is still a matter of debate, however, whether and how the rostral brain is developmentally compartmentalized.

At stages later than 8.5 dpc, *Otx2*$^{otx1/otx1}$ defects were milder than those seen in the Ep$^{-/-}$Ve$^{+/+}$ chimeras. In the latter, at the 6 to 8 somite stage *En* and *Pax2* expressions were confined to a very small domain at the rostralmost tip of the chimera (Rhinn et al., 1998). *Six3* was not expressed even in ventral diencephalon, which corresponds to the future infundibulum, nor was *Hesx1* expressed in Rathke's pouch. By the 12 somite stage the *Hoxa2*-positive r3 was almost the rostral end of the chimeric embryo. In contrast, in *Otx2*$^{otx1/otx1}$ embryos the isthmus and r1/r2 were formed. At 8.5 dpc some structures existed anterior to the *Fgf8*- and *Pax2*-positive isthmus, as evidenced by residual expression of *Emx2*, *Wnt1* and *En1*, as well as knocked-in *Otx1*. Expression of *Six3* and *Fgf8* in ventral diencephalon and *Hesx1* in Rathke's pouch, though faint, were also detected. The 11.5 dpc knock-in embryos had a cephalic furrow and some rostral brain structures, and some structures developed anterior to the pons and cerebellum in prenatal embryos.

Otx1 is weakly expressed at the 1 somite stage and becomes apparent by the 3 somite stage (Simeone et al., 1993; Suda et al., 1997). In subsequent stages *Otx2* may collaborate with *Otx1* to regionalize the rostral brain. In contrast to the Ep$^{-/-}$ Ve$^{+/+}$ chimera, *Otx2* function at this stage might be replaced by knocked-in *Otx1*. At present the only means by which this conclusion can be tested is to examine *Otx1* and *Otx2* double heterozygotes.

We reported previously that *Otx1*$^{+/-}$*Otx2*$^{+/-}$ brain defects

start around the 3 to 6 somite stage; neither *Otx1*$^{+/+}$*Otx2*$^{+/-}$ nor *Otx1*$^{-/-}$*Otx2*$^{+/+}$ showed obvious defects (Suda et al., 1996, 1997). At the 6 somite stage *Otx2* expression in *Otx1*$^{+/-}$*Otx2*$^{+/-}$ embryos was less distinct caudally in the future midbrain region (Suda et al., 1997). Thus *Otx2* expression in forebrain and midbrain appears to be independently regulated, and its expression in midbrain may be autoregulated or regulated by the *Otx1* gene product in a dose-dependent manner. In the midbrain of *Otx1*$^{+/-}$*Otx2*$^{+/-}$ mutants *Wnt1* expression was decreased and *Fgf8* expression remained expanded rather than restricted to the future isthmus (Suda et al., 1997). Double heterozygote defects were pronounced in the mesencephalon and caudal diencephalon, and telencephalon and anterior diencephalon were less affected. These defects were mostly restored in *Otx1*$^{+/-}$*Otx2*$^{+/otx1}$ embryos, suggesting that *Otx2* function at this stage is indeed substitutable by *Otx1*. Recently, Acampora et al. (1998) demonstrated that *Otx1* function at this stage can be substituted for by *otd*, the *Drosophila* orthologue, in a dose-dependent manner; the rescue was less efficient in caudal midbrain. It is important to note that this substitution of *Otx2* function by *Otx1* is in the presence of one *Otx2* allele. It remains to be determined if two *Otx2* alleles are replaceable with *Otx1*.

Establishment of midbrain/hindbrain boundary

The isthmus generates midbrain in a mirror image with an antero-posterior gradient when transplanted in p1 and p2 diencephalon (Marin and Puelles, 1994). Genes such as *Pax2*, *Pax5*, *Fgf8*, *Gbx2*, *En1*, *En2* and *Wnt1* are expressed in a transverse band in the isthmus, and its organizer activity is mimicked by *Fgf8* (Crossley et al., 1996). Initially, the caudal limit of *Otx2* expression in anterior neuroectoderm is obscure and overlaps with *Pax2* and *Fgf8* (Suda et al., 1997). The anterior limit of *Gbx2* expression in hindbrain is also obscure (Wassarman et al., 1997). Shortly after, the caudal limit of *Otx2* expression and the anterior limit of the *Gbx2* expression become distinct, establishing a boundary at which *Pax2* and *Fgf8* expression is confined (Suda et al., 1997; Wassarman et al., 1997). However, it is not yet clear how this boundary, the isthmus, is established. Whether the isthmus plays a role in development of r1 and r2 is also not clear. The establishment of the isthmus occurs after the onset of *Otx1* expression, and *Otx2* may collaborate with *Otx1* in this process (Suda et al., 1997). *Lim1* is not expressed in neuroectoderm nor is *Otx2* expressed in r1 or r2. Nevertheless, null mutants in either gene fail to develop r1 and r2 (Acampora et al., 1995; Matsuo et al., 1995; Shawlot and Behringer, 1995; Ang et al., 1996). Defects observed in *Otx1* and *Otx2* double heterozygotes include dorsal expansion of *Fgf8* expression and expansion of r1 (Suda et al., 1997); a similar phenotype was observed in *Otx1*$^{-/-}$*Otx2*$^{+/-}$ mutants in a different genetic background (Acampora et al. 1997).

Pax2 expression was not seen in our *Otx2*$^{-/-}$ embryos in contrast to recent observations by Rhinn et al. (1998). *Pax2* was, however, induced in the *Otx2*$^{otx1/otx1}$ mutant, as well as in the Ep$^{-/-}$Ve$^{+/+}$ chimeras. Thus we conclude that signals from the visceral endoderm and/or anterior mesendoderm are essential for development of the isthmus and r1/r2. In the Ep$^{-/-}$Ve$^{+/+}$ chimeras, however, r1 and r2 were lost, and r3 became the most anterior structure of the embryo by the 12 somite stage (9.0 dpc), while in *Otx2*$^{otx1/otx1}$ embryos isthmus and r1/2 were maintained. These findings suggest that *Otx* function in neuroectoderm is also required to establish and/or maintain the isthmus, and that signals from the isthmus and/or caudal midbrain are essential for maintenance of r1 and r2.

Otx2 functions in cephalic neural crest cells

Cephalic neural crest cells, a new development in vertebrates, are known to have played essential roles in the evolution of the rostral head (Newth, 1956; Northcutt and Gans, 1983; Langille and Hall, 1988). These cells were responsible for development of the jaw upon transition from agnatha to gnathostomes, one of the most important innovations in vertebrate history. They were also utilized to develop the anterior cranium to support the expanding brain beyond the anterior tip of the notochord. The phenotype of *Otx2*$^{+/-}$ mice revealed the role of *Otx2* in cephalic crest cells (Matsuo et al., 1995). Populations of cephalic neural crest cells are present in an extant agnatha, the lamprey (Horigome et al., 1999), and those cells express an *Otx* cognate (our unpublished result); expression of this cognate was detected in the mesenchyme of the first pharyngeal arch and in mesencephalic crest cells known to give rise to trabecular cartilage (Newth, 1956; Langille and Hall, 1988). Thus the recruitment of new downstream targets is what the *Otx* cognate accomplished with the transition form agnatha to gnathostomes.

Otocephalic defects in *Otx2*$^{+/-}$ mice were largely restored by *Otx1*, and we conclude that *Otx2* function in mesencephalic crest cells is substitutable by *Otx1*. It is, however, not clear why defects are not rescued in all *Otx2*$^{+/otx1}$ mice. Possibly, subtle differences exist in cephalic crest expression of the knocked-in *Otx1* gene compared to expression of endogenous *Otx2*. Alternatively, the affinities of Otx1 protein to target DNA sequences or to cofactors specific to those cells might be lower than those of Otx2 protein, and otocephalic defects might be completely restored at higher *Otx1* dosage.

Divergence into *Otx2* and *Otx1*

Expression patterns of *Amphioxus* and ascidian *Otx* cognates suggest an ancestral role for chordate *Otx* in patterning the anterior mesendoderm and in the involvement of this tissue in induction of anterior neural regions in the overlying ectoderm (William and Holland, 1998). The visceral endoderm, which with the extraembryonic mesoderm forms the visceral yolk sac for maternofetal exchange of nutrients, is, on the other hand, a mammalian innovation. The visceral endoderm may be homologous to the hypoblast in avian species and reptiles, but no directly comparable structure exists in *Xenopus* or zebrafish. Nevertheless, *Otx2* function in the visceral endoderm may represent a universal function throughout vertebrates as a head organizer component. In avian species, the *Otx2* cognate is expressed in the epiblast, the hypoblast, Hensen's node, the anterior mesendoderm and the anterior neuroectoderm (Bally-Cuif et al., 1995). The same is true in *Xenopus* (Pannese et al., 1995; Kablar et al., 1996), but in zebrafish it is *Otx1*-cognates that are expressed at gastrulation in the embryonic shield, the fish organizer, and in the anterior axial mesoderm (Li et al., 1994; Mori et al., 1994; Mercier et al., 1995). In zebrafish anterior neuroectoderm, expression of *Otx1* cognates also precedes that of the *Otx2* cognate. *Xenopus* and zebrafish *Otx* cognates are all expressed maternally, the significance of which is not yet known.

Defects in *Drosophila otd* mutants are restored by *OTX2*, the human *Otx2* homologue, at a higher frequency than by *OTX1* (Leuzinger et al., 1998). Thus it is postulated that the ancestral roles of *Otx/otd* genes are preserved more in *Otx2* cognates in vertebrates, and that *Otx1* evolved new functions required for specific vertebrate developmental programs, which *otd* could not perform. In the $Otx1^{otd/otd}$ mutant, for example, *otd* could not rescue a late *Otx1* function in the lateral semicircular canal that does not exist in lamprey (Acampora et al., 1998). In contrast, the present study suggests that *Otx2* has evolved a new function in establishment of the rostral neuroectoderm. All other *Otx2* functions, in visceral endoderm, anterior mesendoderm, regionalization of anterior neuroectoderm and cephalic neural crest cells, were substitutable by *Otx1*. Such a function, unique to *Otx2*, might involve acquisition of new target sequences that Otx1 protein does not recognize or alterations in affinity to target sequences and/or cofactors with which Otx1 protein interacts weakly. Alternatively, *Otx2* expression may be autoregulated, a regulatory function that cannot be replaced by Otx1 protein, or Otx1 and Otx2 proteins may have different stabilities.

In the gnathostome lineage, when animals diverged into teleost and tetrapod lineages, the ancestor of the former might have retained *Otx1* for head organizer function and used it for the subsequent establishment of the territory of the future forebrain and midbrain; the ancestor of the tetrapod lineage might have retained *Otx2* for these functions. Later in this lineage, *Otx2* might have evolved new functions required for a specific program to establish the tetrapod-unique anterior neuroectoderm, functions that *Otx1* could not perform. Unfortunately, however, no *Otx* cognates have been identified in hagfish, *Petromyzon*, cartilagenous fish, lungfish, coelacanths or teleosts other than zebrafish. To gain further insight into *Otx* cognates and the evolution of the vertebrate rostral brain, it will be important to determine whether lamprey *OtxA* or the zebrafish *Otx1* cognate can substitute for *Otx2* function in mice.

We thank Drs Norimasa Miyamoto and Mika Ohta for their help in production of the knock-in mice. We are indebted to Drs A. P. McMahon, G. Martin, A. Joyner, P. Gruss, D. G. Wilkinson, K. Mahon, G. Schütz, H. Sasaki, B. Herrmann and E. De Robertis for *Wnt1*, *Fgf8*, *En1* and *En2*, *Pax2* and *Six3*, *Krox20*, *Hesx1*, *Fkh2*, *HNF3β*, *Brachyury* and *cer-l* probes for RNA in situ hybridization, respectively. We thank Dr K. Araki for pCAGGS-cre. We are also grateful to the Laboratory Animal Research Center of Kumamoto University School of Medicine for housing the mice. This work was supported in part by grants-in-aid for Specially Promoted Research from the Ministry of Education, Science and Culture of Japan, Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency, Japan, and a Research Grant (9A-1) for Nervous and Mental Disorders from the Ministry of Health and Welfare, Japan.

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