Development 126, 1417-1426 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV1378

Differential transcriptional control as the major molecular event in generating *Otx1*–/– and *Otx2*–/– divergent phenotypes

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Accepted 29 January; published on WWW 3 March 1999

SUMMARY

***Otx1* and *Otx2*, two murine homologs of the *Drosophila orthodenticle (otd)* gene, show a limited amino acid sequence divergence. Their embryonic expression patterns overlap in spatial and temporal profiles with two major exceptions: until 8 days post coitum (d.p.c.) only *Otx2* is expressed in gastrulating embryos, and from 11 d.p.c. onwards only *Otx1* is transcribed within the dorsal telencephalon. *Otx1* null mice exhibit spontaneous epileptic seizures and multiple abnormalities affecting primarily the dorsal telencephalic cortex and components of the acoustic and visual sense organs. *Otx2* null mice show heavy gastrulation abnormalities and lack the rostral neuroectoderm corresponding to the forebrain, midbrain and rostral hindbrain. In order to define whether these contrasting phenotypes reflect differences in expression pattern or coding sequence of *Otx1* and *Otx2* genes, we replaced *Otx1* with a human *Otx2* (*hOtx2*) full-coding cDNA. Interestingly, homozygous mutant mice (*hOtx2*^{1}/*hOtx2*^{1}) fully rescued epilepsy and corticogenesis abnormalities and showed a significant improvement of mesencephalon, cerebellum, eye and lachrymal gland defects. In contrast, the lateral semicircular canal of the inner ear was never recovered, strongly supporting an *Otx1*-specific requirement for the specification of this structure. These data indicate an extended functional homology between OTX1 and OTX2 proteins and provide evidence that, with the exception of the inner ear, in *Otx1* and *Otx2* null mice contrasting phenotypes stem from differences in expression patterns rather than in amino acid sequences.**

Key words: *Otx1*, *Otx2*, Transcriptional control, Cortex, Inner ear, Evolution, Mouse

INTRODUCTION

In vertebrates, a remarkable amount of data has been collected in recent years on the role of gene candidates for the control of developmental programs underlying brain morphogenesis. Most of these genes are the vertebrate homologs of *Drosophila* genes coding for signal molecules or transcription factors (Lemaire and Kodjabachian, 1996; Tam and Behringer, 1997; Rubenstein et al., 1998). Among these, the *orthodenticle* group is defined by the *Drosophila orthodenticle (otd)* and the vertebrate *Otx1*, *Otx2* and *Crx* genes, which contain a bicoid-like homeodomain (Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990; Simeone et al., 1992; Finkelstein and Boncinelli, 1994; Chen et al., 1997; Freud et al., 1997).

Recently, to gain insight into the possibility that a basic genetic program of cephalic development may be conserved between vertebrates and invertebrates, human *Otx* genes have been introduced and overexpressed in *Drosophila otd* mutants and the murine *Otx1* has been replaced with the *Drosophila otd* gene (Acampora et al., 1998a; Leuzinger et al., 1998; Nagao et al., 1998; Sharman and Brand, 1998). Human *Otx1* and *Otx2* genes complement the *otd* defects, allowing the recovery of brain, ventral nerve cord and cephalic defects in flies. Similarly, the *Drosophila otd* gene is able to functionally replace the mouse *Otx1* gene, and fully rescues corticogenesis impairment and epilepsy; it also partially improves eye defects and brain patterning abnormalities detected in *Otx1–/–; Otx2+/–* embryos (Acampora et al., 1997). In contrast, the defective lateral semicircular canal of the inner ear of *Otx1–/–* mice is never recovered (Acampora et al., 1998a).

Comparison of the coding sequences between murine *Otx* and *Drosophila otd* genes indicates that the homology is essentially restricted to the homeodomain, thus suggesting that it might be crucial in selecting at very high stringency the same target sequences, and that the homeodomain-mediated ability to recognize the same target sequence(s) might have been retained in evolution.

In contrast, the role of coding sequences outside the

homeodomain is only poorly understood and it is important to determine whether these regions code for functional domains that are biochemically equivalent, or whether they include a combination of conserved and newly acquired functional specificities. Despite the homeodomain-restricted homology between murine OTX and *Drosophila* OTD gene products, the comparison between murine *Otx1* and *Otx2* coding sequences shows extensive similarities. The similarity is particularly high in the region between the ATG and the end of the homeodomain, while downstream of the homeodomain, the homology to OTX2 is interrupted in OTX1 by blocks of insertions of additional amino acids, including repetitions of histidine and alanine residues (Simeone et al., 1993).

The high homology between *Otx1* and *Otx2* coding sequences, however, does not necessarily imply that they are functionally equivalent. In fact, it is conceivable that the limited amino acid divergence might define *Otx1*- or *Otx2*-specific functional properties.

In mouse, *Otx1* and *Otx2* are activated sequentially during embryonic development. *Otx1* expression is first detected at the 1- to 3-somite stage (8 d.p.c.) throughout the fore- and midbrain neuroepithelium. *Otx2* is already transcribed before the onset of gastrulation in the epiblast and in the visceral endoderm, and at the end of gastrulation is expressed in the axial mesendoderm and rostral neural plate (Simeone et al., 1992, 1993; Ang et al., 1994). During brain regionalization, *Otx1* and *Otx2* are transcribed in largely overlapping expression domains with a posterior border coincident with the mesencephalic side of the isthmic constriction (Simeone et al., 1992; Millet et al., 1996; Acampora et al., 1997) and from 11 d.p.c. onwards only *Otx1* is expressed along the dorsal telencephalon. Furthermore, *Otx1* is transcribed in neurons of deep layers of the adult cerebral cortex (Frantz et al., 1994) and both *Otx1* and *Otx2* are expressed in the olfactory, ocular and acoustic sense organs (Simeone et al., 1993).

Otx1 null mice exhibit spontaneous epileptic seizures and abnormalities affecting primarily the telencephalic cortex, as well as the development of visual and acoustic sense organs (Acampora et al., 1996). *Otx2* null mice die early in embryogenesis, show heavy gastrulation abnormalities and fail to specify rostral neuroectoderm fated to give forebrain, midbrain and rostral hindbrain (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). Therefore, *Otx1* is mainly required in the dorsal telencephalon where *Otx2* is not expressed, and *Otx2* is necessary for proper gastrulation and specification of an early neural plate before 8 d.p.c., when *Otx1* is still off.

In order to determine whether these contrasting phenotypes reflect differences in temporal expression or biochemical activity of OTX1 and OTX2 gene products, we generated a mouse model carrying a human *Otx2 (hOtx2)* full-coding cDNA fragment fused to the 5′ untranslated region of the *Otx1* gene (*hOtx2*^{1}). Epilepsy and corticogenesis defects, due to the absence of *Otx1*, were fully rescued in homozygous *hOtx2*^{1} mice. A significant improvement was also observed for the impairments of the mesencephalon, the eye and the lachrymal gland, while in contrast, defect of the inner ear was never recovered.

Thus, these data provide evidence that, with the exception of the inner ear, *Otx1*–/– and *Otx2*–/– contrasting phenotypes stem from divergent expression patterns. Moreover, the finding that the inner ear defects were not improved strongly supports the existence of an *Otx1*-specific function required in gnathostomes for the correct morphogenesis of this structure.

MATERIALS AND METHODS

Construction of the targeting vector, ES cell transfection and selection of targeted clones

The gene replacement vector was constructed using the same plasmid (pGN) and arms for homologous recombination that was used to produce both *Otx1*–/– (Acampora et al., 1996) and *otd*^{1}/*otd*^{1} mice (Acampora et al., 1998a), but with the human *Otx2* cDNA in place of *lacZ* or *otd* sequences. The *hOtx2* cDNA was previously modified by cloning a 297-bp *Pst*I/*Bam*HI tag fragment corresponding to a region of the λ phage spanning nucleotides 5218-5514, into a *Dra*I site localized in the 3′ UTR of the cDNA, 34 bp downstream of the stop codon in the pCT OTX2 plasmid (Simeone et al., 1993). The *hOtx2* λ tag cDNA was then excised as a *Pvu*II/*Bam*HI fragment spanning a sequence from 49 bp upstream of the methionine to the end of the λ tag and cloned in the final molecule. As in the *Otx1* knock-out and *otd* knock-in vectors, a SV40 polyadenylation signal was present downstream of the cDNA to ensure transcription termination. 15 μg of the targeting vector were linearized by *Not*I digestion and electroporated into 2×10^{7} HM-1 ES cells. Homologous recombinant clones were identified using the same primers as previously described (Acampora et al., 1996, 1998a) (filled arrowheads in Fig. 1A) and confirmed by hybridizing *Hin*dIII-digested genomic DNA with probes A and D (Acampora et al., 1996, 1998a) (hatched boxes in Fig. 1A).

Mouse production and genotyping

Two independent positive clones were injected into C57BL/6 blastocysts and the chimaeric males back-crossed to B6D2F1 females. Genotyping was performed by PCR using two primers specific for the wild-type allele and located in the *Otx1*-deleted sequence (sense primer, AGCAGACACATCGAAACCTTC; antisense primer, CACTTGGGATTTTGCACCCTC) (filled arrowheads in Fig. 1A) and two primers specific for the *hOtx2* cDNA located in the λ tag sequence (sense primer, ATCACCGTCATGCGTGATGC; antisense primer, CGGGATCCCCATAATGCGGCT) (open arrowheads in Fig. 1A).

RNase protection assay and probes

RNase protection was performed as previously described (Simeone et al., 1993). The probe for the *hOtx2* transcript was the *Pst*I/*Bam*HI 297 bp fragment corresponding to the λ tag. The mouse *Otx1* probe was a genomic *Pvu*II/*Bgl*II 166-bp-long fragment containing part of the second exon and second intron. The mouse *Otx2* probe was synthesized from a genomic *Hae*III 142-bp-long fragment containing part of the first exon and first intron.

Western blot analysis

Nuclear extracts were prepared from single or pooled (*n*=3/pool/genotype) 10.5 d.p.c. heads. 20 μg of these extracts and 5 μg of nuclear extracts of HeLa cells transfected with plasmids overexpressing human *Otx1* and *Otx2* cDNAs under a CMV enhancer-promoter (Simeone et al., 1993) were electrophoresed and transferred to nitrocellulose in a standard western blot assay and probed with an antiOTX2 polyclonal antibody (Mallamaci et al., 1996).

Electroencephalographic recordings

The electroencephalographic activity recordings were performed as previously described (Acampora et al., 1996, 1998a).

Histology of brains and sense organs and BrdU labelling

Dissected brains, eyes and inner ears were prepared as previously

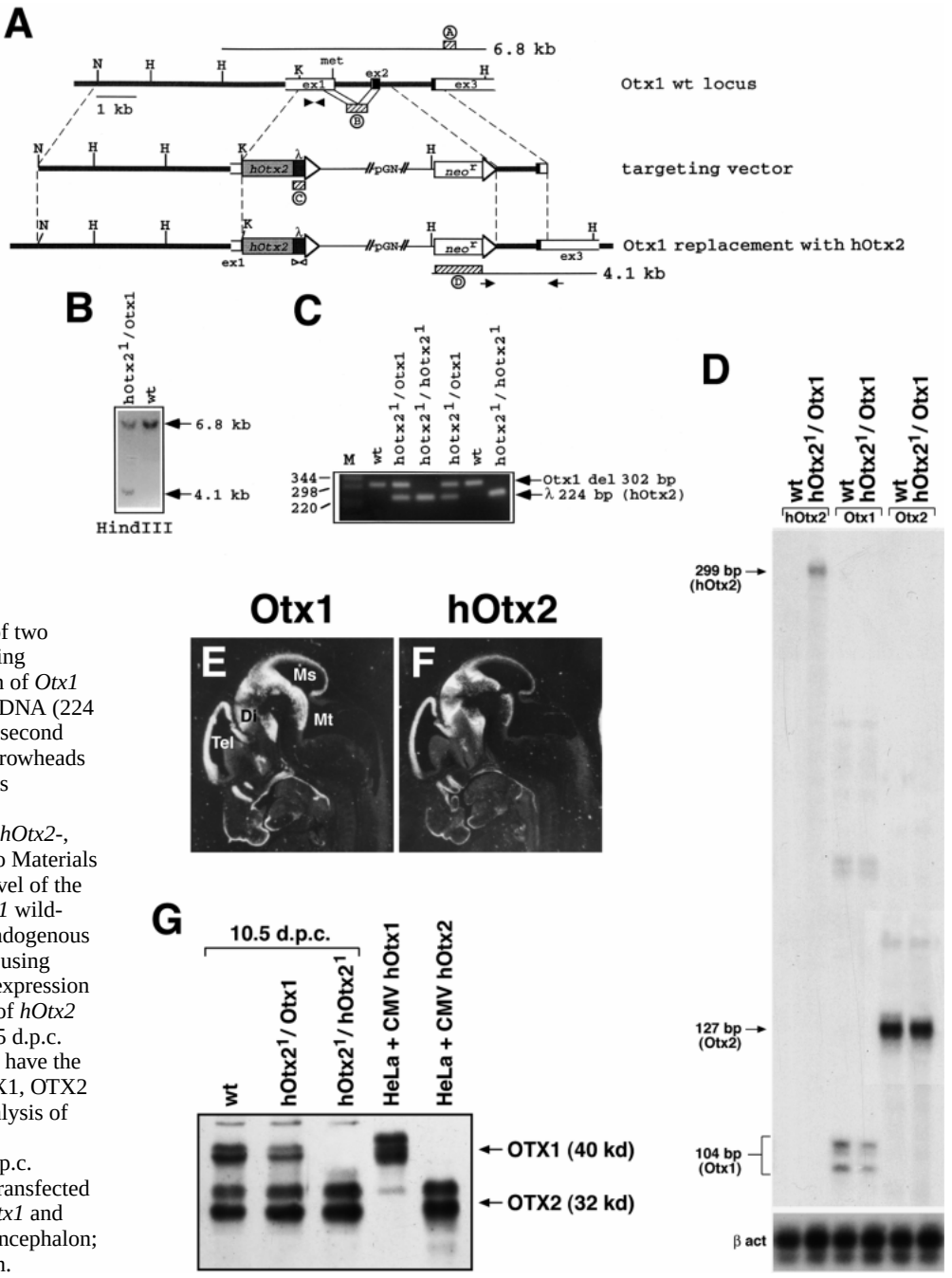

Fig. 1. Targeted replacement of *Otx1* with *hOtx2* cDNA, *hOtx2* expression and hOTX2 protein detection. (A) Targeting vector shown in third line. Fourth line illustrates recombined locus. First and last lines indicate *Hin*dIII fragments (6.8 and 4.1 kb) detected by Southern blot using probes (A-D, hatched boxes) external to the targeting vector or within the *neomycin* gene. N, *Nsi*I; H, *Hin*dIII; K, *Kpn*I. (B) Southern blot analysis of one targeted cell line (*hOtx2*^{1}/*Otx1*) and wild-type (wt) HM-1 cells shows the expected hybridization pattern of *Hin*dIII-digested genomic DNA samples with probe A (A). Only the 4.1 kb fragment is detected with a *neo*-specific probe (probe D) (data not shown). (C) Genotyping of a representative litter from an intercross of two heterozygotes by PCR reaction amplifying fragments specific for the deleted region of *Otx1* (302 bp) and/or the λ tag in the *hOtx2* cDNA (224 bp), using the primers positioned in the second and fourth lines of A (filled and open arrowheads in A). (D) RNase protection experiments performed on RNA from wild-type and *hOtx2*^{1}/*Otx1* 10.5 d.p.c. heads by using *hOtx2*-, *Otx1*- and *Otx2*-specific probes (see also Materials and methods), showing that the RNA level of the *hOtx2* allele is similar to that of the *Otx1* wild-type allele and that the amount of the endogenous *Otx2* transcripts is unaffected. (E,F) By using allele-specific probes B and C (A), the expression pattern of *Otx1* (E) is compared to that of *hOtx2* (F) in adjacent sagittal sections of a 12.5 d.p.c. *hOtx2*^{1}/*Otx1* embryo, showing that they have the same distribution. (G) Detection of OTX1, OTX2 and hOTX2 proteins in western blot analysis of nuclear extracts from single wild-type, *hOtx2*^{1}/*Otx1* and *hOtx2*^{1}/*hOtx2*^{1} 10.5 d.p.c. embryonic heads and from HeLa cells transfected with plasmids overexpressing human *Otx1* and *Otx2* cDNA. Tel, telencephalon; Di, diencephalon; Ms, mesencephalon; Mt, metencephalon.

described (Acampora et al., 1996, 1998a). The mass of freshly dissected brains was also determined. Histological sections (10 μm) were stained with Cresyl Violet (brains) or Haematoxylin and Eosin (eyes). Lachrymal glands were analyzed during the eye dissection. For the fine histological analysis of the cortex, comparable groups of sections centered on the rostral hippocampus (four sections) and posteriorly on the presubicular area (six sections) were selected from 6 wild-type, 6 *Otx1*−/− and 20 *hOtx2*^{1}/*hOtx2*^{1} brains (1-2 months old); histology was as previously reported (Acampora et al., 1996, 1998a). Cell number was determined by counting cell bodies along a cortical area defined by the thickness of the cortex and by a unit length of 200 μm on the ventricular side. For the BrdU labelling, pregnant mice at 9.75, 13.5 and 15.5 d.p.c. were injected intraperitoneally with BrdU solution (50 mg/kg body mass) and killed after 1 hour. After embryo genotyping, BrdU detection was performed according to Xuan et al. (1995). Three embryos for each genotype were scored at 9.75, 13.5 and 15.5 d.p.c. Four comparable sections for each embryo were analyzed at 9.75 d.p.c., while every fourth serial coronal section was selected for a total number of eight sections at 13.5 and 15.5 d.p.c. The fraction of BrdU-positive cells was determined by dividing the number of BrdU-positive nuclei by the total number of nuclei identified in units of neuroepithelium 100 μm in length (Xuan et al., 1995). The proportion of BrdU-positive cells in wild-type embryos was considered 100%.

Paint injection

Some of the inner ears were analyzed by injecting latex paint to the lumen. Heads of 16 d.p.c. embryos were fixed in Bodian's fixative, dehydrated, cleared and injected with 0.1% white latex paint as described (Morsli et al., 1998).

In situ hybridization and probes

In situ hybridization on embryos was performed as described (Hogan et al., 1994). For the *hOtx2^1/Otx1* embryos (Fig. 1E,F) the *hOtx2* probe was the same as previously described for the RNase protection assay, while the murine *Otx1* probe was a 300-bp-long cDNA fragment generated by PCR and corresponding to probe B in Fig. 1A. For the wild-type embryo (Fig. 6A,B) the murine *Otx1* and *Otx2* probes were the same as previously described (Simeone et al., 1993).

Statistical analysis

Analysis of variance was performed according to the program contained in the SYSTAT package (version 5.0) and released by SYSTAT, Inc.

RESULTS

Generation of mice with the *Otx1* gene replaced by a human *Otx2* cDNA

A human *Otx2* cDNA (*hOtx2*) was introduced into a disrupted *Otx1* locus by homologous recombination in embryonic stem (ES) cells. In the *Otx1* targeted locus, a 2.3 kb fragment containing 840 bp of 5′ UTR sequence and the coding region of exons 1 and 2 was deleted and replaced by a full-coding *hOtx2* cDNA, also containing 34 bp of 3′ UTR downstream of the stop codon and 49 bp of 5′ UTR immediately upstream of the methionine. The latter was fused to the remaining part of the *Otx1* 5′ UTR sequence, thus creating a chimaeric transcript. The targeting vector (Fig. 1A) was constructed with the same DNA fragments as previously used, but with a *hOtx2* cDNA-SV40 poly(A) cassette in place of the *lacZ* or *Drosophila otd* sequence (Acampora et al., 1996, 1998a). Human and mouse OTX2 proteins differ by a single conservative (Thr→Ser) substitution in the COOH- terminus and, given the extremely high homology also seen in their nucleotide sequence, a λ tag (see Materials and methods) was introduced immediately downstream of the 3′ UTR sequence of the *hOtx2* cDNA to distinguish transcripts of the transgene allele from that of wild-type *Otx2* gene. This vector was transfected into HM-1 ES cells and, among 180, 9 homologous recombinant clones were identified by PCR and Southern blot analyses (Fig. 1B). Two independent positive clones were then injected into C57BL/6 blastocysts to produce chimaeric mice. Male chimaeras were crossed to B6D2F1 females and scored for germ line transmission of the mutated allele. Heterozygotes (*hOtx2^1/Otx1*) were then intercrossed and genotypes of their litters were determined by allele-specific PCR reactions (Fig. 1C).

Correct expression of *hOtx2* under the transcriptional control of *Otx1* was verified by analyzing the level and the spatial distribution of its transcripts. RNase protection and in situ hybridization experiments were performed by using allele-specific probes (probes B and C in Fig. 1A; see also Materials and methods).

RNase protection assays were performed on RNA extracted from 10.5 d.p.c. wild type and *hOtx2^1/Otx1* embryonic heads, and densitometric scanning of the protected fragments indicated a slight reduction of the *hOtx2* transcript level (approx. 15%) as compared to that of the wild-type *Otx1* allele. In situ hybridization experiments performed on close sections from a 12.5 d.p.c. *hOtx2^1/Otx1* embryo showed that the mRNA distribution of the *hOtx2* allele (Fig. 1F) colocalized with that of the wild-type *Otx1* allele (Fig. 1E). Translation of the *hOtx2* transcripts was monitored by using an OTX2 polyclonal antibody (Mallamaci et al., 1996), recognizing in western blot assays both OTX1 and OTX2 proteins with a very similar efficiency to that shown in HeLa cell extracts transfected with expressing vectors for the human *Otx1* and *Otx2* (Fig. 1G).

10.5 d.p.c. nuclear head extracts from single embryos (Fig. 1G) and a pool of three heads for each genotype (data not shown) were independently assayed in western blots to define the amount of the hOTX2 protein.

As expected, the OTX1 protein was detected in wild-type and *hOtx2^1/Otx1* embryos (Fig. 1G). In wild type, as revealed by densitometric comparison between OTX1 and OTX2 gene products, the amount of the OTX1 protein was half of that corresponding to OTX2. Therefore, in *hOtx2^1/hOtx2^1* embryos a maximum increase of approx. 50% was expected for the OTX2 protein. Our data showed that in *hOtx2^1/Otx2* embryos the OTX2 increase was of approx. 20% and in *hOtx2^1/hOtx2^1* of approx. 35% (Fig. 1G).

hOtx2^1/hOtx2^1 mice rescued epilepsy and postnatal lethality detected in *Otx1–/–* mice

hOtx2^1/hOtx2^1 mice were generated at the expected frequency and the postnatal lethality observed in *Otx1–/–* mice (approx. 30%) was rescued to a normal level (approx. 4%).

As previously shown (Acampora et al., 1996), *Otx1–/–* mice exhibited epileptic seizures in hippocampus and cortex (Fig. 2A) and abnormal high-speed-turning behavior.

All the *hOtx2^1/hOtx2^1* mice showed a moderate-speed-turning behavior and lacked the phenotypic characteristics of both focal and generalized seizures.

To confirm the rescue of epilepsy, electroencephalograms were performed on ten *hOtx2^1/hOtx2^1*, four wild type and four *Otx1–/–* 2-month-old mice.

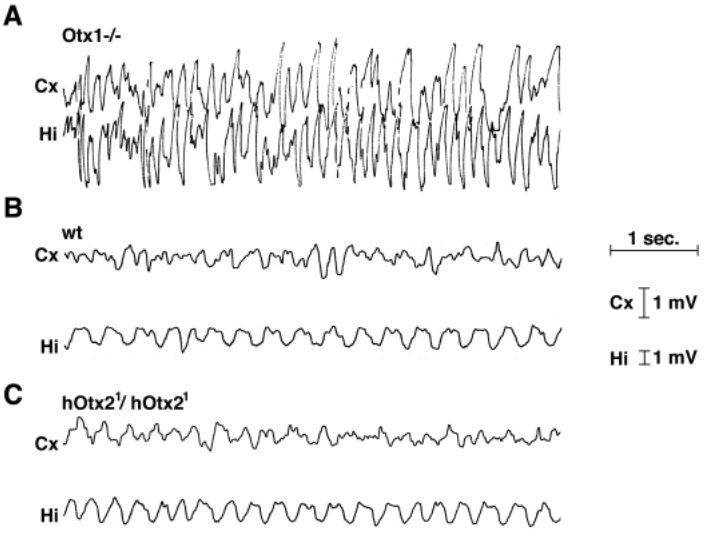

Fig. 2. *hOtx2^1/hOtx2^1* mice recover from epilepsy. (A-C) Representative electroencephalogram recordings showing an *Otx1–/–* epileptic seizure (A) with high-voltage spiking activity in hippocampus (Hi) and cortex (Cx), corresponding to the convulsive behavior, while in wild-type (B) and *hOtx2^1/hOtx2^1* (C) mice a normal electrical activity was recorded in all cases analyzed.

The electroencephalogram recordings were performed in two rounds of 45 minutes each on 2 different days. As compared to wild-type mice (Fig. 2B), only *Otx1–/–* mice exhibited prolonged seizures in hippocampus and cortex (Fig. 2A), while in all of the *hOtx2¹/hOtx2¹* mice a normal electric activity was recorded (Fig. 2C).

hOtx2¹/hOtx2¹ mice rescued *Otx1–/–* brain abnormalities

Otx1–/– brains (Fig. 3B) were reduced in size and mass by about 25-30% compared to wild type (Fig. 3A) and displayed a number of anatomo-histological abnormalities (Acampora et al., 1996).

In fact, in *Otx1–/–*, the dorsal telencephalic cortex showed an overall reduction that was more evident in the temporal and perirhinal areas. The reduction in thickness and cell number was also accompanied by a disorganization of cortical cell-layers of the temporal and perirhinal areas where the sulcus rhinalis was hardly recognizable. Moreover, the relative extent of the cortical regions located dorsal versus those ventral to the presumptive sulcus rhinalis was abnormal and a clear reduction of the dorsal region was observed (Acampora et al., 1996, 1998a).

To determine whether the hOTX2 gene product mediated the recovery of cortical abnormalities caused by the absence of OTX1, we analyzed the anatomy and histology of 20 *hOtx2¹/hOtx2¹* brains from 2-month-old mice.

As compared to wild type and *Otx1–/–* (Table 1 and Fig. 3A,B), 16 of the *hOtx2¹/hOtx2¹* brains (Table 1 and Fig. 3C) showed the normal size and mass, while only one of them displayed a size similar to that of *Otx1–/–* mice, and three of them were of intermediate size and mass (Table 1 and data not shown). In frontal sections, as compared to *Otx1–/–* (Fig. 3E,H), all the *hOtx2¹/hOtx2¹* brains, including that with the strongest size reduction (data not shown), increased the thickness of the telencephalic cortex (Fig. 3F,I) and restored the correct relative size of the regions located dorsally and ventrally to the presumptive sulcus rhinalis (compare black and red lines of Fig. 3I to those in Fig. 3G and H, respectively). However, it is noteworthy that the sulcus rhinalis was not clearly identified in *Otx1–/–* brains and, thereby, its presumptive position was also deduced by a careful histological analysis. On this basis it was assigned immediately dorsal to the entorhinal and/or piriform cortices whose typical histology was easily recognized. As previously mentioned, in *Otx1–/–* brains the reduction in dorsal telencephalic cortex was likely due primarily to a reduction in cell number that was particularly evident in the presumptive temporal and perirhinal areas where a disorganization in the cortical cell layers was also detected. We analyzed in detail the cortical histology of all the 20 *hOtx2¹/hOtx2¹* brains previously mentioned.

All of them, including those with an intermediate size,

Table 1. Brain mass

	Brain number	Brain mass in mg (range)	Mean±s.d.
Wild type	6	500-580	525±20
Otx1–/–	6	330-380	356±19
	16	480-550	510±24
hOtx2¹/hOtx2¹	3	420-460	443±21
	1	390	

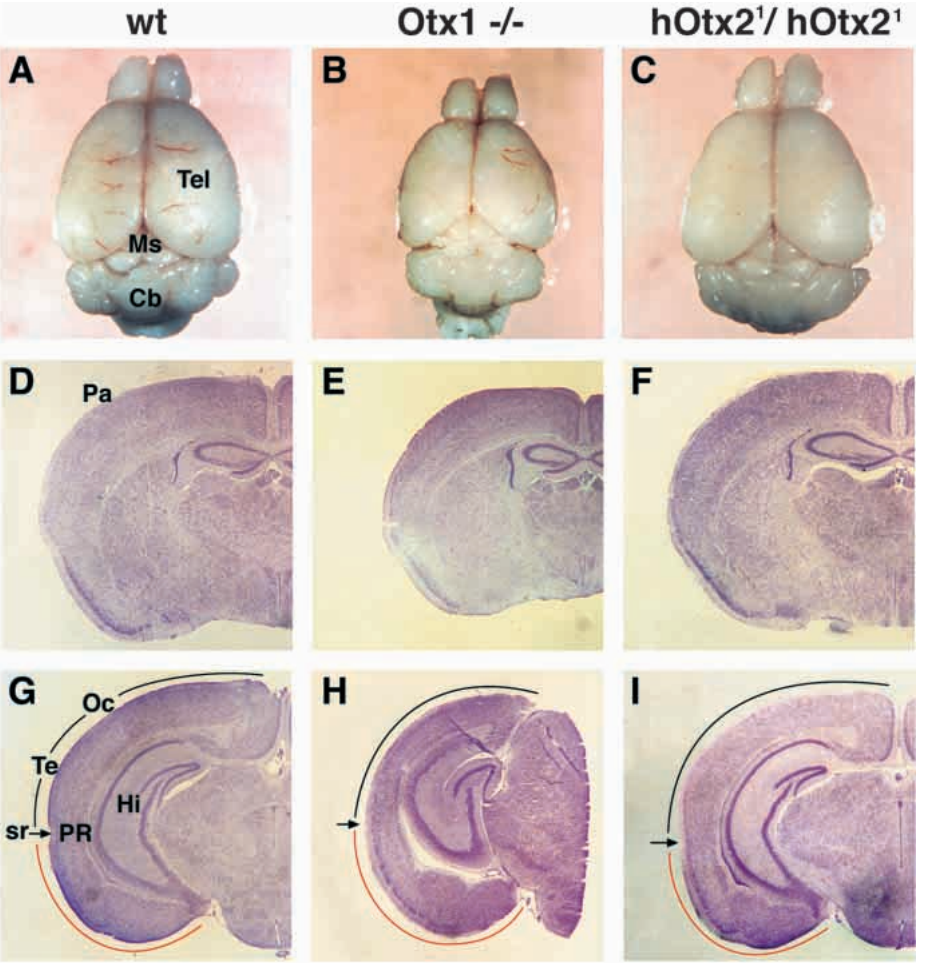

Fig. 3. *hOtx2¹/hOtx2¹* brains rescue normal size and recover corticogenesis abnormalities. (A-C) Dorsal view of wild-type (A), *Otx1–/–* (B) and *hOtx2¹/hOtx2¹* (C) brains showing the normal size of the *hOtx2¹/hOtx2¹* brain. (D-I) Frontal sections of wild type (D,G), *Otx1–/–* (E,H) and *hOtx2¹/hOtx2¹* (F,I) indicate that the cortical impairments and the abnormal morphology of the hippocampus are largely recovered in *hOtx2¹/hOtx2¹* mice. Pa, Oc, Te and PR, parietal, occipital, temporal and perirhinal areas, respectively; Hi, hippocampus; Cb, cerebellum; sr, sulcus rhinalis; other abbreviations as in the previous figures. Black and red lines define the regions located dorsally and ventrally to the presumptive sulcus rhinalis, respectively.

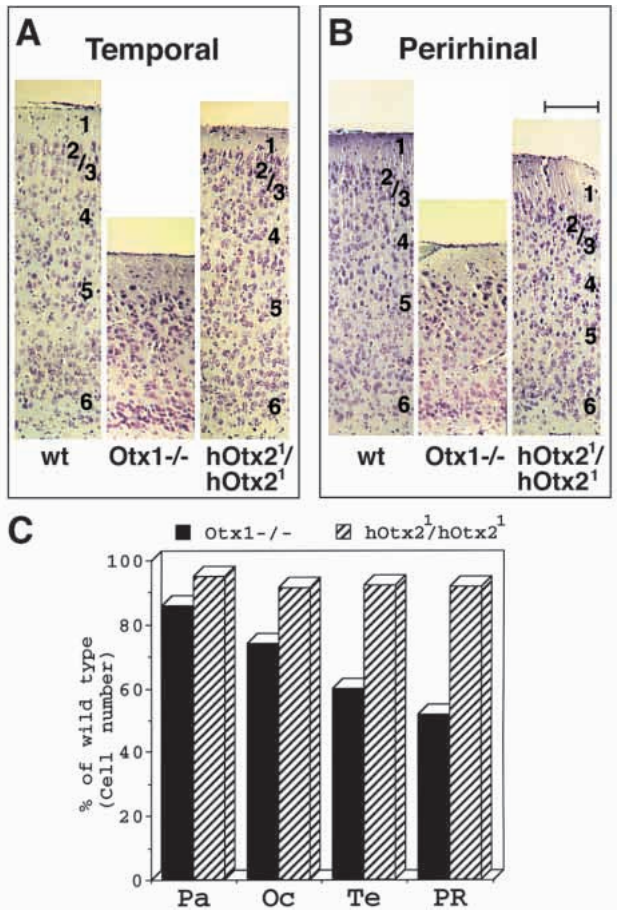

Fig. 4. Histology and cell number in the dorsal cortex of *hOtx2¹/hOtx2¹* brains. (A,B) As compared to wild type and *Otx1–/–*, the temporal (A) and perirhinal (B) cortices of *hOtx2¹/hOtx2¹* mice recovered an apparently normal thickness and cell-layer organization that are strongly impaired in *Otx1–/–* mice. (C) *Otx1–/–* and *hOtx2¹/hOtx2¹* cell numbers in parietal, occipital, temporal and perirhinal areas are reported as percentage of wild type. Abbreviations as in the previous figures. Bar, 100 μm.

showed an apparently normal organization of cortical cell layers and an increased cell number (Fig. 4A-C) even though the cell number in the perirhinal and temporal cortices of brain with intermediate size showed a 15% reduction as compared to wild type (data not shown). However, the mean value of the cell number detected within the different telencephalic areas of the 20 *hOtx2¹/hOtx2¹* brains was comparable to that of wild-type mice (Fig. 4C). In Table 2, the lowest and highest cell number as well as the mean value and its standard deviation were reported for the perirhinal, temporal and occipital areas of the three genotypes. Moreover, the analysis of variance among the three genotypes and the orthogonal matches of *Otx1–/–* versus wild type, + *hOtx2¹/hOtx2¹*, and of wild type versus *hOtx2¹/hOtx2¹*, were also shown (Table 3). From this analysis it appeared that while difference in cell number between *Otx1–/–* and wild type + *hOtx2¹/hOtx2¹* were highly significant, the difference between wild type and *hOtx2¹/hOtx2¹* became less and less significant moving the analysis from the occipital to the perirhinal cortex (Table 3).

Furthermore, *Otx1–/–* mice were also affected in other brain districts such as the hippocampus, mesencephalon and cerebellum.

In fact in *Otx1–/–* brains the hippocampus was shrunken, the superior and inferior colliculi of the mesencephalon were enlarged and the cerebellum displayed abnormal foliation in approx. 70% of them (Acampora et al., 1996). The presence of these abnormalities was monitored in *hOtx2¹/hOtx2¹* brains. As compared to wild-type and *Otx1–/–* brains, in *hOtx2¹/hOtx2¹* the hippocampus appeared morphologically normal (compare Fig. 3I to G,H); the mesencephalon returned to a normal size in 30% (*n*=6) (compare Fig. 5C to A,B) and to an intermediate size in 45% of them (data not shown); the cerebellum retained a normal foliation in 50% of the cases (compare Fig. 5F to D,E).

Cell proliferation in the telencephalic neuroepithelium of *hOtx2¹/hOtx2¹* mice

We used a short pulse of bromodeoxyuridine (BrdU) labelling to study cell proliferation in the developing neuroepithelium of

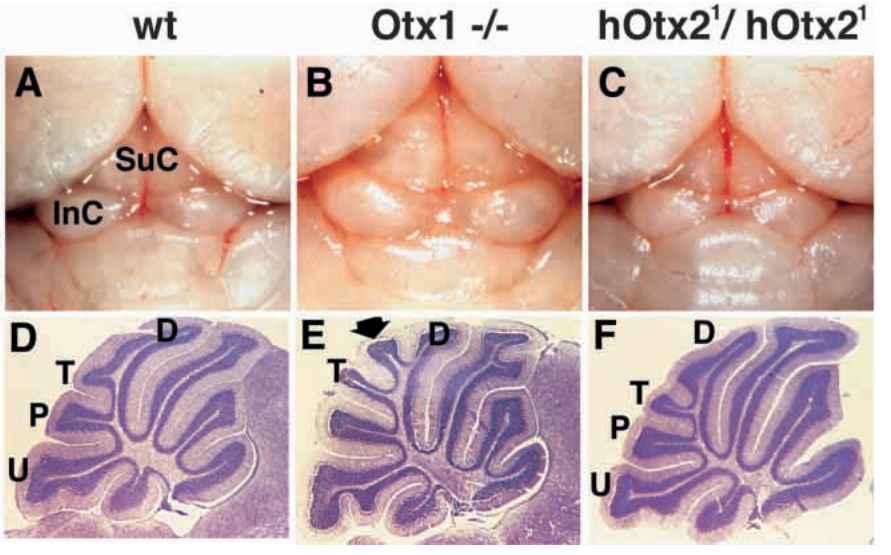

Fig. 5. Size and morphology of the mesencephalon and cerebellar foliation are recovered in a significant number of *hOtx2¹/hOtx2¹* mice. (A-F) As compared to wild type (A,D) the mesencephalon of *Otx1–/–* (B) mice displays increased size of the superior and inferior colliculi, and the cerebellum (E) an abnormal foliation with the presence of an additional lobule (arrow in E) in 70% of them; in *hOtx2¹/hOtx2¹* embryos the mesencephalon (C) shows a normal size in 45% of the brains scored and the cerebellum (F) a normal foliation in 50% of them. SuC and InC, superior and inferior colliculi; D, declivus; T, tuber; P, pyramis; U, uvula.

Table 2. Cell number in the perirhinal, temporal and occipital cortices

	Brain number	Cell number (range)	Mean±s.d.
Perirhinal			
Wild type	6	290-370	321±37
Otx1–/–	6	165-202	181±14
*hOtx2^1/hOtx2^1**	20	254-350	293±31
Temporal			
Wild type	6	300-362	332±21
Otx1–/–	6	192-230	209±22
*hOtx2^1/hOtx2^1**	20	263-350	301±39
Occipital**			
Wild type	6	305-400	339±33
Otx1–/–	6	230-308	267±35
*hOtx2^1/hOtx2^1**	20	264-388	305±35

*The reported *hOtx2^1/hOtx2^1* brains include 16 brains with an apparently normal size, 3 brains with an intermediate size and 1 brain with a size similar to that of *Otx1–/–* mice.

**The occipital cortex corresponds to the lateral occipital area adjacent to the temporal cortex.

Table 3. Analysis of variance of neuronal cell numbers

	Variance among the three genotypes		Orthogonal matches: *Otx1–/–* versus (wt + *hOtx2^1/hOtx2^1*)		Orthogonal matches: Wild type versus *hOtx2^1/hOtx2^1*	
	F	*P*	F	*P*	F	*P*
Perirhinal	22.17	≤10^{-4}	43.9	≤10^{-4}	2.47	0.132
Temporal	21.39	≤10^{-4}	42	≤10^{-4}	3.66	0.07
Occipital	6.64	0.005	9.9	0.004	4.876	0.037

F, analysis of variance; P, probability.

hOtx2^1/hOtx2^1 embryos at 9.75, 13.5 and 15.5 d.p.c. (see Materials and methods).

At the earliest stage analyzed (9.75 d.p.c.) the vast majority of cells was actively proliferating, defining the progenitor pool that contributed to the expansion of the ventricular zone. Between 11 and 17 d.p.c. of mouse corticogenesis, neurons born early will occupy deeper layers and neurons born later will occupy more superficial layers (McConnell, 1995). It has been previously reported that the failure of dorsal cortex to grow normally in *Otx1–/–* mice might be primarily caused by impairment of proliferative potentialities of neuronal precursors around 9.75 d.p.c. (Acampora et al., 1998a). Interestingly, at this stage, as deduced in adjacent frontal sections throughout the telencephalon of wild-type embryos, *Otx1* (Fig. 6A) expression domain filled the dorsal territorial gap where *Otx2* (Fig. 6B) was not transcribed. In fact, *Otx1* was expressed all along the presumptive dorsal telencephalic neuroepithelium and *Otx2* in the most medio-dorsal area and in the basal neuroepithelium (arrows in Fig. 6A,B). In *hOtx2^1/hOtx2^1* and *hOtx2^1/Otx1* embryos, by using allele-specific probes (probes B and C in Fig. 1A), the distribution of *hOtx2* transcripts is identical to that of the endogenous *Otx1* locus (data not shown). At the same stage, the proliferative activity of the dorsal and basal neuroepithelium of *hOtx2^1/hOtx2^1* (Fig. 6E,F) embryos was compared to that of wild type (Fig. 6C,F) and *Otx1–/–* (Fig. 6D,F) embryos. Similarly to homozygous mice replacing *Otx1* with the *Drosophila otd* gene (Acampora et al., 1998a), *hOtx2^1/hOtx2^1*

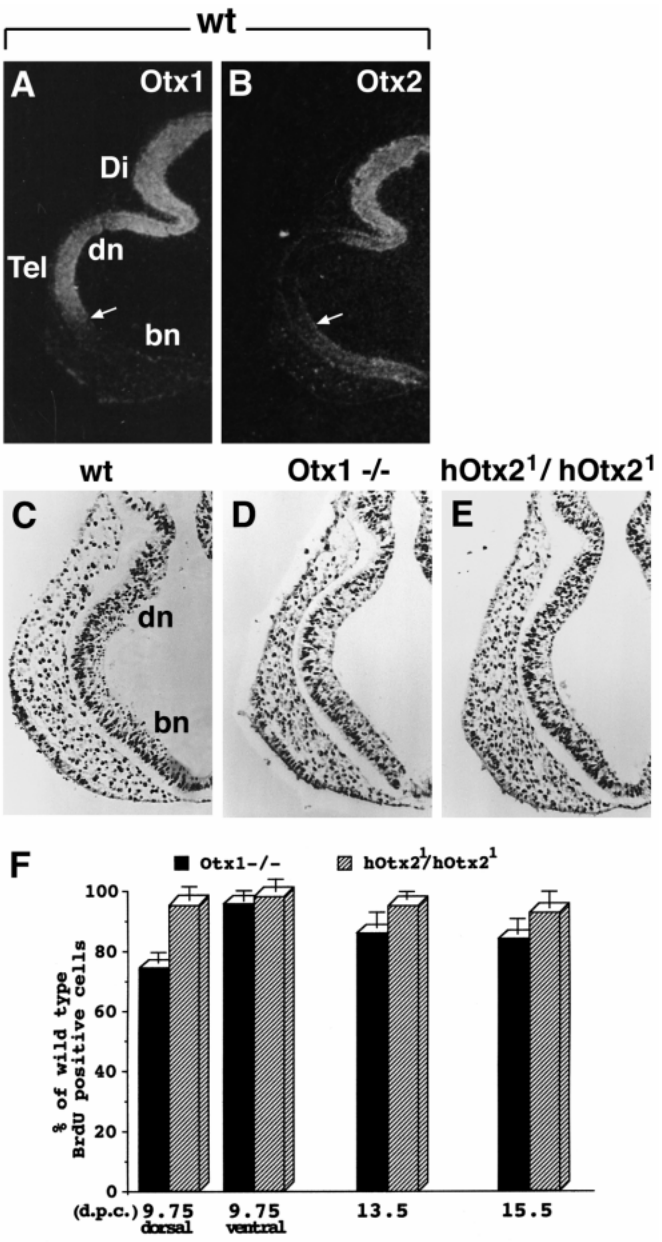

Fig. 6. Rescue of proliferation in the dorsal telencephalon of *hOtx2^1/hOtx2^1* embryos. (A,B) *Otx1* (A) and *Otx2* (B) expression patterns in frontal sections of 9.75 d.p.c. wild-type embryos. The arrows point to the boundary region between the *Otx1* and *Otx2* expression domains. (C-E) BrdU-positive cells in frontal sections of 9.75 d.p.c. embryos showing that, as compared to wild type (C), the decrease of BrdU-positive cells detected in the presumptive dorsal neuroepithelium of *Otx1–/–* embryos (D) is recovered in *hOtx2^1/hOtx2^1* (E) embryos. (F) *Otx1–/–* and *hOtx2^1/hOtx2^1* BrdU-positive cells at 9.75, 13.5 and 15.5 d.p.c. are reported as a percentage of those detected in wild-type embryos of the same stages along the same unit of neuroepithelium (see also Materials and methods). The number of BrdU-positive cells between wild type and *hOtx2^1/hOtx2^1* does not show relevant differences, while a significant decrease is detected in the dorsal telencephalon of *Otx1–/–* embryos mainly at 9.75 d.p.c. Note that in the presumptive ventral telencephalon of 9.75 d.p.c. *Otx1–/–* embryos, the number of BrdU-positive cells is comparable to the wild type. Percentages in F are means ± s.e.m. dn, dorsal neuroepithelium; bn, basal neuroepithelium; other abbreviations as in previous figures.

embryos also showed improved proliferative activity in a region of the dorsal neuroepithelium (Fig. 6E,F) where only *Otx1* is expressed (Fig. 6A,B). At the subsequent stages analyzed (13.5 and 15.5 d.p.c.), the proliferative activity of *hOtx2^1/hOtx2^1* embryos was not affected (Fig. 6F and data not shown).

hOtx2^1/hOtx2^1 mice did not recover *Otx1–/–* inner ear defects

Additional abnormalities were reported in *Otx1–/–* mice affecting the eye, the inner ear and the lachrymal and Harderian glands (Acampora et al., 1996). In fact the ciliary process in the eye (Fig. 7B), the lachrymal and Harderian glands (Fig. 7E) and the lateral semicircular canal of the inner ear (Fig. 7H) were not identified. As compared to wild type (Fig. 7A,D,G) and *Otx1–/–* (Fig. 7B,E,H) in *hOtx2^1/hOtx2^1* (Fig. 7C,F,I) the ciliary process was present in 70% of the eyes scored (n=40) (Fig. 7C) and the lachrymal and Harderian glands in approx. 75% of the cases (n=50) (Fig. 7F), while in contrast, the lateral semicircular canal of the inner ear was not identified in any of the 45 inner ears analyzed (Fig. 7I). There were additional inner defects in *Otx1–/–* mutants, which were only partially rescued by *hOtx2*. Those data will be presented separately.

The finding that *hOtx2*, like the *otd* gene (Acampora et al., 1998a), did not recover the lateral semicircular canal of the *Otx1–/–* inner ear strongly suggests that the ability to specify this structure may represent a specific property of the OTX1 gene product. Nevertheless, it is necessary to consider whether the absence of the lateral semicircular canal might be also contributed by the quantitative reduction (approx. 30%) of the hOTX2 protein, even though it is noteworthy that in double heterozygous (*Otx1+/–; Otx2+/–*) embryos (n=9), the lateral semicircular canal was always correctly detected (data not shown).

DISCUSSION

Gene duplication and modification of the regulatory control of gene expression may greatly contribute to the increased complexity of the body plan. These events appear particularly relevant in the vertebrate evolution from protochordates (Garcia-Fernandez and Holland, 1994; Holland et al., 1994; Williams and Holland, 1998). In fact, drastic evolutionary modifications in copy number and/or expression pattern might likely represent the most rapid and efficient mechanisms to confer morphological changes during embryonic development. Hence, gene duplication may allow the duplicated gene to acquire new specific function(s) either retaining or lacking ancestral properties, and similarly, modification of the regulatory control of gene expression may establish new expression patterns that might alter pre-existing cell fates by generating new specialized cellular functions. Murine OTX1 and OTX2 proteins share high amino acid sequence homology, despite a decrease in this homology in the region located downstream of the homeodomain, mainly because of the presence of insertions of blocks of amino acid residues in OTX1 (Simeone et al., 1993).

In protochordates (urochordates and cephalochordates) only a single *Otx* gene has so far been identified, and strong evidence based on phylogenetic analysis supports the idea that in amphioxus the *Amphi Otx* gene is the most recent ancestor of the vertebrate *Otx1* and *Otx2* genes (Williams and Holland, 1998).

The *Amphi Otx* gene is expressed early in the anterior mesendoderm and neuroectoderm, while at a later stage it is transcribed in a specific group of cells in the anterior neural tube (Williams and Holland, 1996).

This suggests that, following gene duplication, *Otx2* retains its early expression pattern, which is probably related to its oldest role in patterning the mesendoderm, and *Otx1* acquires a new expression pattern since it disappears from the murine gastrula and becomes stably transcribed in the dorsal telencephalon. Functionally speaking, what does this gene duplication event imply? Do *Otx1* and *Otx2* have different

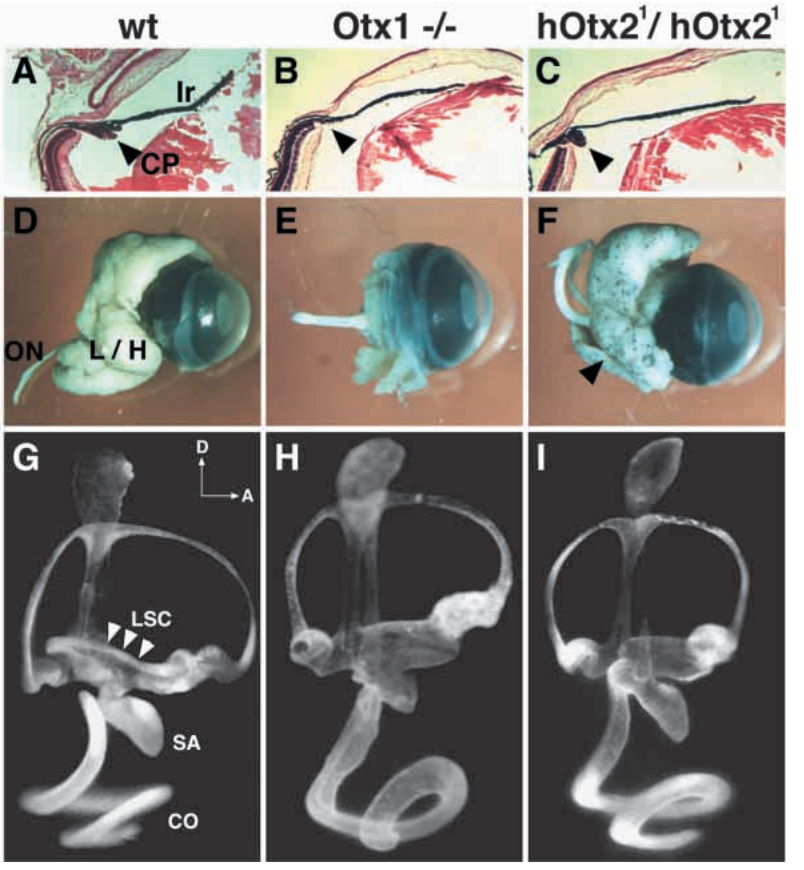

Fig. 7. *hOtx2^1/hOtx2^1* mice recover the ciliary process and the lachrymal/Harderian gland complex, but retain the absence of the lateral semicircular canal in the inner ear. (A-I) As compared to wild type (A,D,G) *Otx1–/–* mice lack the ciliary process in the eye (B, arrowhead), the lachrymal/Harderian gland complex (E) and the lateral semicircular canal of the inner ear (H); in most of *hOtx2^1/hOtx2^1* mutant mice the ciliary process (C, arrowhead) and the lachrymal/Harderian gland complex (F, arrowhead) are rescued, while interestingly, the lateral semicircular canal is never recovered (I). Ir, iris; L/H, lachrymal/Harderian gland complex; ON, optic nerve; CP, ciliary process; LSC, lateral semicircular canal; SA, sacculus; CO, cochlea; D and A, dorsal and anterior.

roles? Are these distinct roles derived from modification of their expression control or, alternatively, from their limited amino acid divergence?

Gene duplication events have been reported for many genes and one of the most remarkable examples is the HOX gene family (Holland et al., 1994; Holland and Garcia-Fernandez, 1996). A likely consequence of the increased genomic complexity may be an increase in the number of molecular interactions among the gene products of the duplicated functions. Such an increase in molecular interactions may contribute toward modifying relevant morphogenetic processes that in turn may confer a change in shape and size of the body plan as well as in the generation of cell-types with new developmental potentialities. On this basis, *Otx* gene duplication and subsequent or contemporary modification of regulatory control might have contributed to the evolution of the mammalian brain (e.g. by increasing the extent of neuroectodermal territory recruited to form the brain). This event might involve an improvement of proliferative activity of early neuronal progenitors (Acampora et al., 1998a and this work) and/or the positioning of the mes-met boundary (Acampora et al., 1997; Suda et al., 1997). Additional property(ies) may be conferred to the duplicated gene by altering its coding sequence. Thus, the limited amino acid divergence between OTX1 and OTX2 might underlie modifications of their functional properties.

Null mice for *Otx1* and *Otx2* have been generated to gain insight into their specific roles (Acampora et al., 1995, 1996, 1997; Matsuo et al., 1995; Ang et al., 1996; Suda et al., 1997). Although their analyses highlight the *Otx1* and *Otx2* roles during embryonic development, it does not help to define whether the differences between their expression patterns have been more relevant than those between their coding sequences in generating *Otx1–/–* and *Otx2–/–* divergent phenotypes. To approach this question, we replaced the *Otx1* coding sequence with that of the human *Otx2* gene. We showed that the recombined locus was correctly expressed under the *Otx1* transcriptional control and that, as compared to the endogenous OTX1 gene product, the hOTX2 protein was approx. 30% lower. Nevertheless, $hOtx2^{1}/hOtx2^{1}$ mice fully rescued epilepsy and cortical impairments and, though with a reduced efficiency, also showed improvements of mesencephalon, cerebellum, eye and lachrymal/Harderian gland complex abnormalities. Interestingly, the mice never recovered the lateral semicircular canal of the inner ear.

Therefore, these data provide in vivo evidence that, with the exception of the inner ear, *Otx1* and *Otx2* share an extended functional homology. We have already reported that in mice replacing *Otx1* with the *Drosophila otd* gene (Acampora et al., 1998a) yields a phenotype very similar to that of $hOtx2^{1}/hOtx2^{1}$ mice and, similarly to $hOtx2^{1}/hOtx2^{1}$ mice, they never rescue the inner ear defect.

Thus, previous and present data strongly support the possibility that the ability to specify the lateral semicircular canal of the inner ear might represent a unique property of the *Otx1* coding sequence.

Interestingly, the inner ear phenotype hints at possible evolutionary implications. In fact, the absence of this structure in *Otx1–/–* mice might represent a back evolutionary mutation and suggests when the *Otx1*-type gene appeared in evolution. The inner ear of lower agnates such as mixinoides shows only one semicircular canal, two ciclostomes and three gnathostomes. The last to be created is the lateral semicircular canal in gnathostomes (Fritsch et al., 1998; Torres and Giraldez, 1998). Only one *Otx* gene has been identified so far in protochordates (urochordates and cephalochordates) (Wada et al., 1996; Williams and Holland, 1996), while there are at least two *Otx* genes in lamprey (agnates) (Ueki et al., 1998). However, even though one of these two genes is clearly related to *Otx2*, the other cannot be unambiguously related to the murine *Otx1* (Ueki et al., 1998). Therefore, it may be speculated that in lamprey the *Otx1* ancestor has not yet duplicated or, alternatively, that it is still evolutionary unstable.

In this context it has been reported that *Otx1*-related genes show a higher rate of sequence evolution compared with that of *Otx2* (Williams and Holland, 1998). It will be interesting, however, to test whether *Otx* genes from protochordates rescue the *Otx1* requirement for the inner ear defect.

We therefore propose that an unambiguous *Otx1*-related gene, having the ability to specify the lateral semicircular canal of the inner ear, its more exclusive property, was established in lower gnathostomes.

Since no obvious homology exists between OTD and OTX1 proteins in the non-homeodomain regions, the rescue observed with the *Drosophila otd* gene suggested a crucial role of the homeodomain. On this basis, since the human *Otx2* is much more similar to *Otx1* than the *otd* gene, it would be expected that it should be even more efficient in rescuing *Otx1* requirements.

Interestingly, the recovery observed in $hOtx2^{1}/hOtx2^{1}$ mice is essentially the same reported for otd^{1}/otd^{1} mice, thus suggesting that the coding regions outside the homeodomain are not crucial for the specification of *Otx* gene functions or, more likely, that functional properties defined by coding regions external to the homeodomain are present also in the *Drosophila otd* gene but, due to the high sequence divergence, these are difficult to identify.

Finally, our data also lead us to predict a possible recovery of the *Otx2* phenotype with either the *Otx1* or the *Drosophila otd* genes. Previous reports in *Drosophila* (Leuzinger et al., 1998; Nagao et al., 1998; Sharman and Brand, 1998) and preliminary results in mouse indicate that visceral endoderm-restricted translation of *Otx1* and *otd* in this tissue rescues the *Otx2* requirements for the specification of the early anterior neural plate and proper gastrulation (Acampora et al., 1998b and unpublished results). Therefore, the data presented here and those previously reported (Hanks et al., 1995; Acampora et al., 1998a; Leuzinger et al., 1998; Nagao et al., 1998; Sharman and Brand, 1998) support the possibility that during pre-early vertebrate evolution most of the *Otx1* roles were decided by modifying its transcriptional control, while only a few specific properties were gained by altering its coding sequence (e.g. the lateral semicircular canal of inner ear).

We thank H. Reichert, S. Martinez and M. Gulisano for helpful discussions, E. Parisi for great support with the statistical analyses, M. Terracciano for technical support and A. Secondulfo for manuscript preparation. This work was supported by the Italian Telethon Program, the Italian Association for Cancer Research (AIRC), the 'CNR Target Project on Biotechnology', the 'MURST-CNR Biotechnology Programme Legge 95/95' and the EC BIOTECH programme.

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