

Otx genes are required for tissue specification in the developing eye

Juan Ramon Martinez-Morales¹, Massimo Signore^{2,3}, Dario Acampora^{2,3}, Antonio Simeone^{2,3}
and Paola Bovolenta^{1,*}

¹Instituto Cajal, CSIC, Dr Arce 37, Madrid 28002, Spain

²MRC Centre for Developmental Neurobiology, New Hunt's House, 4th Floor, King's College London, Guy's Campus, London Bridge, London SE11 9RT, UK

³International Institute of Genetics and Biophysics, CNR, Via Marconi, 12, 80125 Naples, Italy

*Author for correspondence (e-mail: bovolenta@cajal.csic.es)

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SUMMARY

Patterning of the vertebrate eye appears to be controlled by the mutual regulation and the progressive restriction of the expression domains of a number of genes initially co-expressed within the eye anlage. Previous data suggest that both *Otx1* and *Otx2* might contribute to the establishment of the different eye territories. Here, we have analysed the ocular phenotype of mice carrying different functional copies of *Otx1* and *Otx2* and we show that these genes are required in a dose-dependent manner for the normal development of the eye. Thus, all *Otx1*^{-/-}; *Otx2*^{+/-} and 30% of *Otx1*^{+/-}; *Otx2*^{+/-} genotypes presented consistent and profound ocular malformation, including lens, pigment epithelium, neural retina and optic stalk defects. During embryonic development, optic vesicle infolding was severely altered and the expression of pigment epithelium-specific genes, such as *Mitf* or *tyrosinase*, was lost. Lack of pigment epithelium specification was associated with an

expansion of the prospective neural retina and optic stalk territories, as determined by the expression of *Pax6*, *Six3* and *Pax2*. Later in development the presumptive pigment epithelium region acquired features of mature neural retina, including the generation of Islet1-positive neurones. Furthermore, in *Otx1*^{-/-}; *Otx2*^{+/-} mice neural retina cell proliferation, cell differentiation and apoptotic cell death were also severely affected. Based on these findings we propose a model in which *Otx* gene products are required for the determination and differentiation of the pigment epithelium, co-operating with other eye patterning genes in the determination of the specialised tissues that will constitute the mature vertebrate eye.

Key words: *Otx1*, *Otx2*, Eye, Pigment epithelium, Optic vesicle patterning, Mouse

INTRODUCTION

Vertebrate eye development proceeds through the co-ordinated activation of morphogenetic programs based on inductive interactions among tissues of different embryonic origin. The eye primordium comprises the optic vesicle (OV), a lateral protrusion of the diencephalic wall, and the lens placode, a thickening of the ectoderm overlying the vesicle. The progressive determination of this primordium further originates functionally specialised eye tissues: the optic stalk (OS), the neural retina (NR), the retinal pigment epithelium (RPE) and the crystalline. Recent studies have demonstrated that all these eye developmental processes are controlled by a complex network of regulatory genes many of which have been highly conserved throughout evolution (Jean et al., 1998; Gerhing and Ikey, 1999). In particular, the specification of eye compartments appears to be controlled by the mutual regulation and the progressive restriction of the expression domains of a number of transcriptional regulators initially coexpressed within the eye anlage (Macdonald et al., 1995; Schwarz et al., 2000; Nguyen and Arnheiter, 2000). This is the case for instance of *Pax2*, *Pax6*, or *Mitf* which are initially expressed throughout the OV and later become restricted to the

OS, NR and RPE, respectively (Nornes et al., 1990; Walter and Gruss, 1991; Torres et al., 1996; Nakayama et al., 1998; Schwarz et al., 2000). Analysis of *Pax2* and *Pax6* null mutant mice demonstrated that the activity of both genes is necessary not only to establish the identity but also the border between OS and optic cup (OC; Hill et al., 1991; Torres et al., 1996; Schwarz et al., 2000). Furthermore, in mice with mutation in the *Mitf* locus, patches of the RPE lose their characteristics, and acquire features of NR (Nakayama et al., 1998; Bumsted and Barnstable, 2000; Nguyen and Arnheiter, 2000).

Expression analysis of *Otx2*, one of the two vertebrate homologous of the *Drosophila* cephalic gap gene *orthodenticle* (*otd*; Finkelstein and Perrimon, 1990; Simeone et al., 1992; Simeone et al., 1993) had led to the proposal that *Otx2* might also participate in the establishment of the eye territories (Bovolenta et al., 1997). Even though little is known about the expression of *Otx1* during vertebrate eye development, both *Otx1* and *Otx2* are initially expressed throughout the OV (Simeone et al., 1993). Later *Otx2* becomes specifically restricted to the dorsal portion of the vesicle, the presumptive RPE territory (Bovolenta et al., 1997). After that regional specification of the eye is achieved, a second wave of *Otx2* expression was found also in the NR. In particular, OTX2

was localised to postmitotic neuroblasts committed to both neuronal and glia cell types (Bovolenta et al., 1997). In spite of the critical distribution of *Otx* genes in the developing eye, analysis of *Otx1* and *Otx2* mutant mice has only given limited information about how these genes function during eye formation. *Otx1*^{-/-} mice lack ciliary processes and lachrymal glands (Acampora et al., 1996), whereas *Otx2*^{+/-} mice are either normal (Acampora et al., 1995) or lack lens, cornea and iris or display anophthalmia or microphthalmia (Matsuo et al., 1995), depending on the genetic background. A complete analysis of *Otx2* activity in the eye is impaired by the high embryo lethality due to gastrulation defects and by the severe head malformation present in *Otx2* null homozygotes (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996).

To understand *Otx* functions in general it is important to consider the particular functional interaction between the two genes. Although *Otx* genes are responsible for different aspects of anterior vertebrate brain morphogenesis, a different transcriptional control rather than divergent protein activities appears to be responsible for the distinct defects found in *Otx1* and *Otx2* null mice (Simeone, 1998; Acampora and Simeone, 1999). Thus, the proteins encoded by the two genes have, at least in part, similar biochemical activities, since replacement of *Otx1* gene with *Otx2* cDNA and vice versa could partially rescue the respective knock-out phenotype (Acampora et al., 1998; Acampora et al., 1999; Suda et al., 1999). In agreement with this idea, the two *Otx* genes appear to cooperate in some aspects of vertebrate brain morphogenesis since a minimum level of OTX protein is required to specify the isthmic organizer position and, therefore, the further patterning of the anterior brain (Acampora et al., 1997; Suda et al., 1997). Because a similar synergistic action of *Otx* genes is necessary for the normal development of the mouse inner ear (Morsli et al., 1999), we have postulated that cooperation of these genes might be also necessary for eye development.

Here we have analysed the eye phenotype of *Otx*-deficient mice. We show that in all *Otx1*^{-/-}; *Otx2*^{+/-} and in 30% of *Otx1*^{+/-}; *Otx2*^{+/-} genotypes, the development of all the tissues derived from the OC were severely affected. The failure of the RPE differentiation was the major phenotypic malformation observed in these mutant embryos. Lack of RPE was associated with an expansion of the prospective NR and OS territories. Based on these findings we propose a model in which *Otx* proteins are required for the determination of the RPE, cooperating with other eye patterning genes in the specification of the different tissues derived from the vertebrate OV.

MATERIALS AND METHODS

Mouse embryos

Otx-deficient mice were generated by crossing *Otx1*^{+/-}; *Otx2*^{+/-} males with *Otx1*^{+/-}; *Otx2*^{+/+} females in a BL6/DBA2 background and the offspring were further genotyped as described previously (Acampora et al., 1995; Acampora et al., 1996; Acampora et al., 1997). Wild-type BALB/c mice were used in *Otx2*-PAX2 double labelling experiments. The day of the appearance of the vaginal plug was considered as embryonic day (E)0.5.

Probes

Digoxigenin-labelled antisense RNA probes for the mouse *tyrosinase*, *Mitf*, *Prox1* and *Otx2* genes were prepared as described (Beerman et

al., 1992; Hodgkinson et al., 1993; Oliver et al., 1993; Bovolenta et al., 1997). The ³⁵S-labelled RNA probes for *Otx1*, *Otx2*, *Pax2*, *Pax6* and *Six3* genes were generated as reported (Acampora et al., 1997).

In situ hybridisation

Experiments using ³⁵S-labelled RNA probes were performed as previously described (Wilkinson, 1992). Digoxigenin-labelled hybridisations were carried out on tissue sections as described (Bovolenta et al., 1997). Briefly, embryos were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.3) at 4°C overnight, and then cryoprotected by immersion in 30% sucrose solution in PB. Cryostat sections (18 µm) were mounted on 2% 3-aminopropyltriethoxy-silane-coated slides, air dried, and treated with methanol for 10 minutes at room temperature. After rinses in phosphate-buffered saline (PBS), sections were prehybridised for 1 hour at 65°C in 50% formamide buffer, incubated with digoxigenin probes for 16 hours at 65°C, and further washed at the same temperature. After incubation in 0.5% Boehringer blocking reagent solution for 1 hour, sections were incubated with AP-coupled anti-digoxigenin antibody (Roche) diluted 1:5000. After the AP reaction was revealed, sections were coverslipped and analysed in a Leica DMR compound microscope.

Antibodies

Polyclonal antibodies against Islet1 (generated in guinea-pig), phosphorylated-histone H3 (P-H3; Upstate) PAX2 (Zymed) and α-A-crystallin were used at 1:5000, 1:1000, 1:2000 and 1:3000 dilution respectively. Polyclonal antiserum against the mouse OTX2 protein (Mallamaci et al., 1996) was used at 1:2000. Monoclonal antibodies against phosphorylated β-tubulin (Tuj1) and MITF (Neomarkers) were used at 1:4000 and 1:1000 dilution, respectively.

Immunocytochemistry

Embryo heads were fixed by immersion in 4% paraformaldehyde in PBS overnight at 4°C. After washing in PBS, the tissue was equilibrated in 30% sucrose in PBS, embedded in OCT compound and sectioned on a cryostat at 16 µm. Alternatively, embryos were dehydrated, embedded in Paraplast and sectioned on a microtome (Leica) at 16 µm. Tissue was permeabilised in methanol containing 10% DMSO for 10 minutes, rinsed in PBS and blocked for 1 hour in 10% FCS in PBS containing 0.1% Tween (PBTF). Sections were incubated with each primary antibody diluted in PBTF overnight at 4°C. Primary antibodies were localised with either goat anti-mouse, rabbit (Jackson ImmunoResearch) or guinea-pig (Chemicon International) biotin-conjugated IgG, followed by either Cy3 (Amersham-Pharmacia) or peroxidase-conjugated streptavidin (Jackson ImmunoResearch). Fluorescently immunolabelled sections were mounted with PBS/glycerol and analysed with a laser scanning confocal imaging system (TCS 4D; Leica). Peroxidase-coupled samples were revealed using NovaRed substrate kit (Vector Laboratories), dehydrated, coverslipped and analysed in a Leica DMR compound microscope.

Statistical analysis

Quantitative data are expressed as mean ± s.e.m. Significant differences among groups were evaluated by an unpaired *t* test (GraphPad Prism).

RESULTS

Otx1 is expressed in the dorsal OV and in the presumptive RPE

The detailed expression of *Otx2* during eye development has been reported previously (Simeone et al., 1993; Bovolenta et al., 1997), in contrast to that of *Otx1* which has been poorly

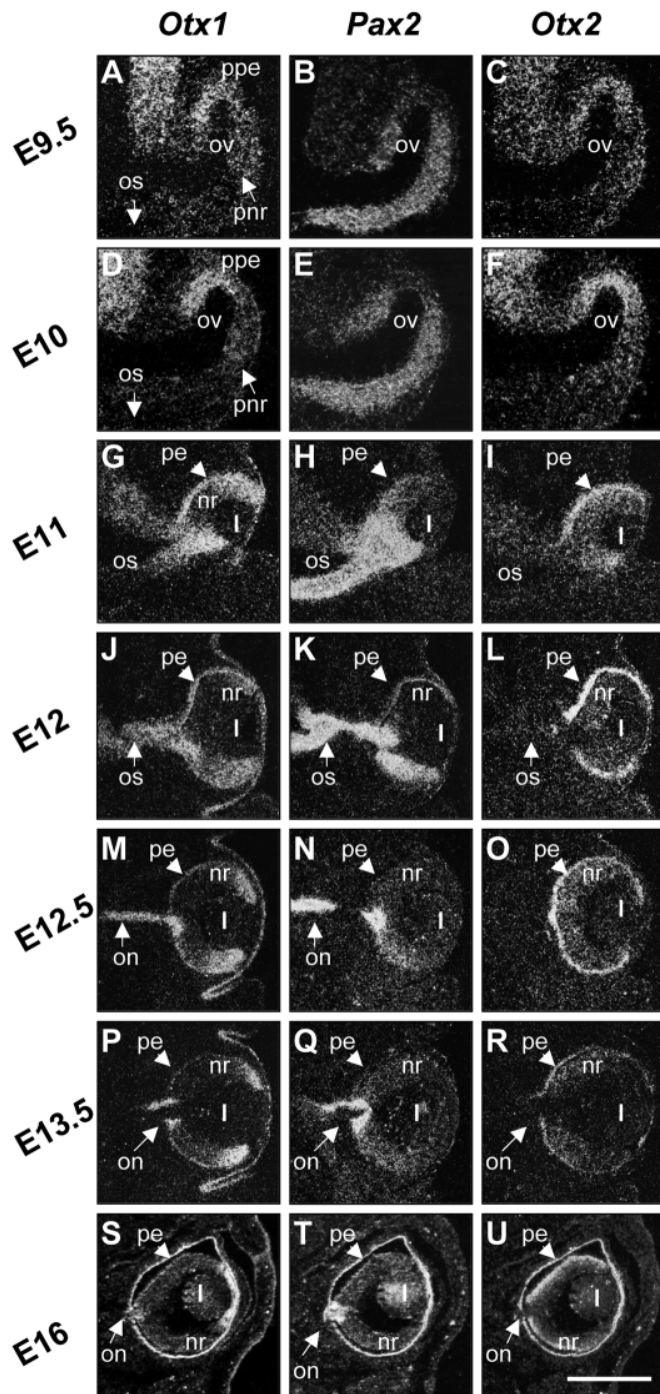


Fig. 1. Expression pattern of *Otx1* as compared to that of *Otx2* and *Pax2* in the developing mouse eye. Dark-field images of frontal adjacent paraffin sections of wild-type embryos at different stages of development hybridised with radioactive probes for *Otx1*, *Pax2* and *Otx2*, as indicated in the figure. Note how at early stages, *Otx1* (A,D) and *Otx2* (C,F) signals overlap and are complementary to that of *Pax2* (B,E). Note how *Otx1* (M,P) but not *Otx2* (O,R) expression extends only to the dorsal portion of the optic nerve at later stages, partially overlapping with *Pax2* (N,Q). The signal observed in the RPE in (T) is due to pigment granules and not to hybridised probe. I, lens; nr, neural retina; on, optic nerve; os, optic stalk, ov, optic vesicle; pnr, presumptive neural retina; ppe, presumptive pigment epithelium; pe, retinal pigment epithelium. Bar: 200 μ m (A-F), 300 μ m (G-I), 400 μ m (J-O), 500 μ m (P-R), and 700 μ m (S-U).

described. Because this was a necessary piece of information to understand the phenotype of *Otx*-deficient mice, we have analysed *Otx1* distribution by situ hybridisation in mouse embryos. *Otx1* transcript localisation was compared on consecutive sections with that of *Otx2* and *Pax2*, dorsal and ventral markers of the OV, respectively (Nornes et al., 1990; Bovolenta et al., 1997). At E9.5-E10 *Otx1* mRNAs were restricted to the dorsal portion of the OV. This distribution was overlapping with that of *Otx2* and complementary to that of *Pax2*, which was confined to the vesicle ventral half (Fig. 1A-F). Later (E10.5-E16), *Otx1* was clearly localised to the presumptive RPE (Fig. 1J,M,P,S), as was *Otx2*. At these stages *Pax2* expression was progressively limited to the ventral OS (Fig. 1; Nornes et al., 1990). *Otx1*, but not *Otx2* expression was also evident in the peripheral NR and in the dorsal half of the developing optic nerve (Fig. 1J-U). A similar *Otx1* expression pattern was observed in the developing chick eye (not shown).

***Otx*-deficient mice present gross eye malformations**

Mice carrying different functional copies of *Otx1* and *Otx2* genes were generated by mating *Otx1*^{+/-}; *Otx2*^{+/-} double heterozygous males with *Otx1*^{+/-}; *Otx2*^{+/+} females, as described (Acampora et al., 1997). A total of 68 embryos were analysed at different developmental stages (Table 1). Among these, all *Otx1*^{-/-}; *Otx2*^{+/-} embryos (*n*=24) presented gross eye

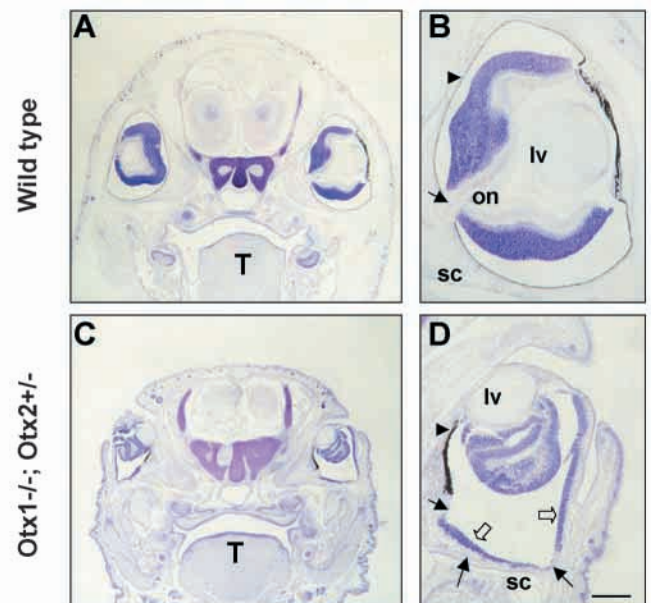


Fig. 2. Ocular phenotype of *Otx1*^{-/-}; *Otx2*^{+/-} neonatal mice. Frontal paraffin sections of wild-type (A,B) and *Otx1*^{-/-}; *Otx2*^{+/-} (C,D) neonatal mice were stained with Cresyl Violet to assess morphology. Images in A and C were taken from sections at equivalent axial levels. B and D are high magnification views of part of A and C, respectively. Note that in mutants both eyes show very similar alterations. The lens is rotated about 90° dorso-nasally, NR is folded toward the lens and fibres exit the eye in aberrant positions (arrows in B,D points to fibre tracts). RPE is totally absent but for a patch of pigmented tissue (arrowhead in D). The majority of the RPE has differentiated as a NR (open arrows in D). lv, lens vesicle; nr, neural retina; on, optic nerve; pe, retinal pigment epithelium; sc, sclera; T, tongue. Bar: A,C, 900 μ m; B,D, 300 μ m.

malformations that ranged from unilateral anophthalmia to severe abnormalities in the lens, NR and RPE (Table 1; Fig. 2C,D). Among the other genotypes, 30% of the *Otx1*^{+/-}; *Otx2*^{+/-} embryos (*n*=13) showed similar but less severe malformations (Table 1). The remaining viable genotypes did not presented gross eye malformations (Table 1), but as previously reported (Acampora et al., 1996), mild ciliary defects were observed in *Otx1*^{-/-} mice from E15 onwards.

Fig. 2 illustrates the typical ocular phenotype of *Otx1*^{-/-}; *Otx2*^{+/-} neonatal mice (mice die soon after birth), as determined by histological analysis in Cresyl Violet stained sections. As compared to wild type (Fig. 2A,B), mutant mice consistently presented alterations in four different eye structures (Fig. 2C,D). The lens was either absent (Fig. 8B; Table 1) or reduced in size (Fig. 7) and was usually mislocated with a 90° rotation. The NR was dysplastic and folded. Retinal ganglion cell (RGC) axons that normally coalesce in the optic nerve head exited the eye in disorganised bundles following aberrant trajectories around the external surface of the eye (Fig. 2D). The last and most dramatic alteration was, however, the almost complete absence of a morphologically recognisable RPE. This tissue was replaced by a thickened neuroepithelium with a NR-like morphology (Fig. 8B). In several embryos, a dorsal patch of pigmented tissue (Fig. 2D) constituted the only remnant of the RPE.

Therefore, mice with the minimal dosage of OTX proteins compatible with viability presented a clear failure in the proper patterning of the eye.

Gene expression is altered in the OV of *Otx*-deficient mice

To understand the morphological and molecular mechanisms responsible for the abnormal ocular phenotype described above, we have analysed the expression of different genes at critical stages of eye development. In mice, OV are well developed at E9.5. At this stage, OV outpocketing appeared normal in all *Otx* mutants analysed, including those with the lower *Otx* dosage (Fig. 3). We next tested for possible alterations in the expression of genes that may contribute to the regionalisation of the OV, such as *Pax2*, *Pax6* and *Six3* (Hill et al., 1991; Torres et al., 1996; Oliver et al., 1995; Bovolenta et al., 1998; Schwarz et al., 2000). The distribution of these genes, together with that of the remaining *Otx2* allele(s), was analysed in the different *Otx*-deficient genotypes by in situ hybridisation on consecutive sections (Fig. 3). In wild type and *Otx1*^{-/-} mice (Figs 3A,B, 1C,F), the expression of *Otx2* was limited to the dorsal half of the vesicle in the presumptive RPE territory, as previously reported (Simeone et al., 1993; Bovolenta et al., 1997). In contrast, in *Otx1*^{+/-}; *Otx2*^{+/-} and *Otx1*^{-/-}; *Otx2*^{+/-} embryos *Otx2* mRNA was uniformly distributed at low level throughout the entire vesicle, including its ventral portion (Fig. 3C-E). Similarly, *Pax2* transcripts, which normally become restricted to the ventral half of the OV at this stage (Figs 1B,E, 3F,G; Nornes et al., 1990), were still expressed in more dorsal locations in embryos carrying a single functional copy of *Otx2* (Fig. 3H-J). None of the *Otx*-deficient genotypes presented significant differences

Table 1. *Otx* gene dosage effects on eye development

Genotypes*	Number of embryos analysed	% Gross eye malformations
<i>Otx2</i> ^{+/-} ; <i>Otx2</i> ^{+/-}	10	0
<i>Otx1</i> ^{+/-} ; <i>Otx2</i> ^{+/-}	8	0
<i>Otx1</i> ^{-/-} ; <i>Otx2</i> ^{+/-}	7	0‡
<i>Otx1</i> ^{+/-} ; <i>Otx2</i> ^{+/-}	6	0
<i>Otx1</i> ^{+/-} ; <i>Otx2</i> ^{+/-}	13	30
<i>Otx1</i> ^{-/-} ; <i>Otx2</i> ^{+/-}	24	100§

**Otx2*^{-/-} embryos die during or shortly after gastrulation (Acampora et al., 1995; Matsuo et al., 1996; Ang et al., 1996).
 ‡Neural retinas of *Otx1*^{-/-}; *Otx2*^{+/-} mice from E15 onwards presented ciliary defects, but no pigment epithelium or lens malformations.
 §12.5% *Otx1*^{-/-}; *Otx2*^{+/-} mice lacked lens unilaterally. 20.8% these mice had unilateral anophthalmia.

in *Pax6* or *Six3* ocular expression as compared to wild-type embryos (Fig. 3K-T).

Mitf, a helix-loop-helix-zipper transcription factor expressed in the eye field (Hodgkinson et al., 1993), becomes specifically localised to the presumptive RPE early in eye development (Nguyen and Arnheiter, 2000; Fig. 4D). As shown by immunocytochemical detection of both proteins, MITF and OTX2 distribution overlaps in the dorsal OV (Fig. 4A,D). In *Otx1*^{+/-}; *Otx2*^{+/-} and *Otx1*^{-/-}; *Otx2*^{+/-} embryos instead, MITF localisation was not restricted to the outer layer of the OV (Fig. 4E,F), following the abnormal expression pattern of *Otx2* (Figs 4B,C, 3C-E).

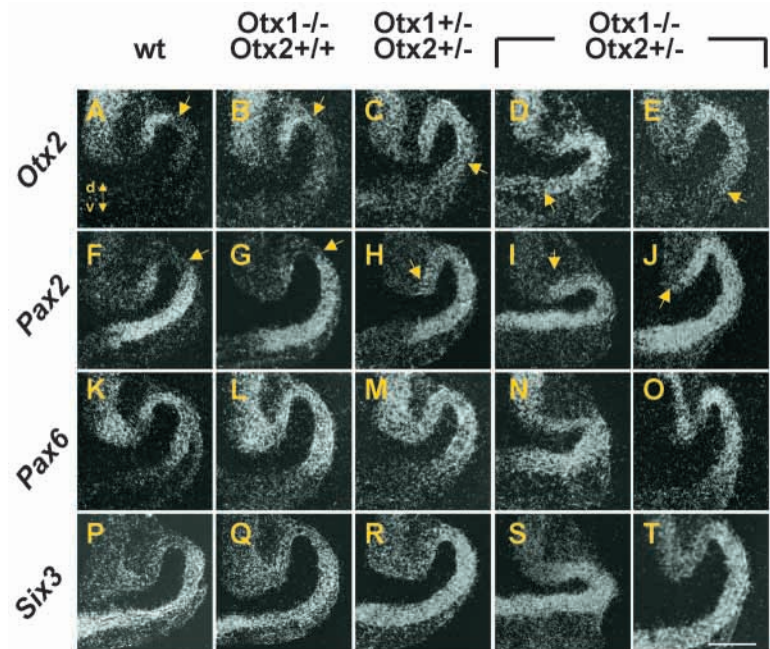


Fig. 3. Comparison of *Otx2*, *Pax2*, *Pax6* and *Six3* expression domains in the OV of *Otx*-deficient mice. Dark-field images of transverse paraffin sections of the OV of wild-type and *Otx* mutant embryos hybridised with radioactive probes for *Otx2*, *Pax2*, *Pax6* and *Six3*, as indicated. Note how in all genotypes OV have evaginated normally and *Pax6* and *Six3* expression is similar in all genotypes. In contrast, expression of *Pax2* and of the remaining *Otx2* allele is extended respectively ventrally and dorsally in both *Otx1*^{+/-}; *Otx2*^{+/-} (C, H) and *Otx1*^{-/-}; *Otx2*^{+/-} (D-E, I-J) as compared to wild-type (A, F) or *Otx1*^{-/-} embryos (B, G). Arrows in A-J indicate the limits of expression in the OV. d, dorsal; v, ventral. Bar, 200 μm.

Altogether these data indicate that a single copy of *Otx2* is sufficient for both the specification of the eye field in the anterior neural plate and the evagination of the OV from the diencephalic wall. A low level of OTX proteins is however insufficient to achieve the proper segregation of the expression domain of vesicle patterning genes, such as *Pax2* or *Mitf*.

RPE determination fails in *Otx*-deficient mice

Soon after evagination, the OV begins to fold over the lens vesicle. The OC, which is fully formed in mice at E12.5, is the result of these morphogenetic movements. At this stage the outer layer of the cup will start to acquire characteristics of the future RPE forming a monolayer of cuboid cells that present the restricted expression of both *Otx1* and *Otx2* (Figs 1, 4G). In addition, the outer layer of the cup express other RPE-specific genes such as *Mitf* (Fig. 4J) and *tyrosinase*, a melanin synthesing enzyme (Fig. 4M; Beerman et al., 1992).

The folding of the OV into OC was severely altered in all *Otx1*^{-/-}; *Otx2*^{+/-} and in 33% of the *Otx1*^{+/-}; *Otx2*^{+/-} embryos analysed. In these mutants, most of the outer layer of the developing OC appeared as a thickened neuroepithelium (Figs 4K,L,N,O, 5B,D,F,H) similar to that of the prospective NR. In this abnormal layer of the cup, OTX2 as well as *Mitf* and *tyrosinase* mRNAs were completely undetectable (Fig. 4). In both mutants only small patches of the outer layer acquired RPE characteristics. Even though cells did not form a cuboid epithelium (insets in Fig. 4G-I), co-expression of OTX2, *Mitf* and *tyrosinase* was always found in these patches (Fig. 4).

In wild-type mice, the transition from OV to OC results in the restriction of the expression of *Pax6*, *Six3* and *Pax2* to specific compartments. Thus, *Pax6* and *Six3* transcripts become localised to the presumptive NR (Fig. 5A,C; Walther and Gruss, 1991; Oliver et al., 1995; Bovolenta et al., 1998), while those of *Pax2* to the OS territory (Fig. 5E; Nornes et al., 1990; Torres et al., 1996). In *Otx1*^{-/-}; *Otx2*^{+/-} embryos the expression of *Pax6* and to a lower level that of *Six3* was abnormally extended to the entire OC (Fig. 5B,D). Similarly, the expression of *Pax2* and that of its product are limited to the entire ventral region of the developing OC (Fig. 6A-C) and later only to the stalk region of the fully formed cup (Figs 5E, 6B,D), both *Otx2*-negative territories (Fig. 6A,B). In contrast, as already suggested by analysis at E9.5, *Otx1*^{-/-}; *Otx2*^{+/-} embryos presented an enlargement of the *Pax2* domain which was first expanded dorsally in E10.5 OC (Fig. 6E) and subsequently extended over the RPE territory at E12.5 (Fig. 6F).

Altogether the expression profiles of these genes suggest that in *Otx*-deficient mice RPE specification does not occur. The outer layer of the OC acquires, instead, features of the prospective NR from one side and that of the OS from the other.

Lens development abnormalities in *Otx*-deficient mice

Lens formation proceeds through successive phases of specification, which involves the interaction of the

presumptive lens ectoderm (PLE) with the newly evaginated OV (Grainger 1996; Grainger et al., 1997; Jean et al., 1998). Both *Otx1* and *Otx2* genes are expressed in the vertebrate PLE

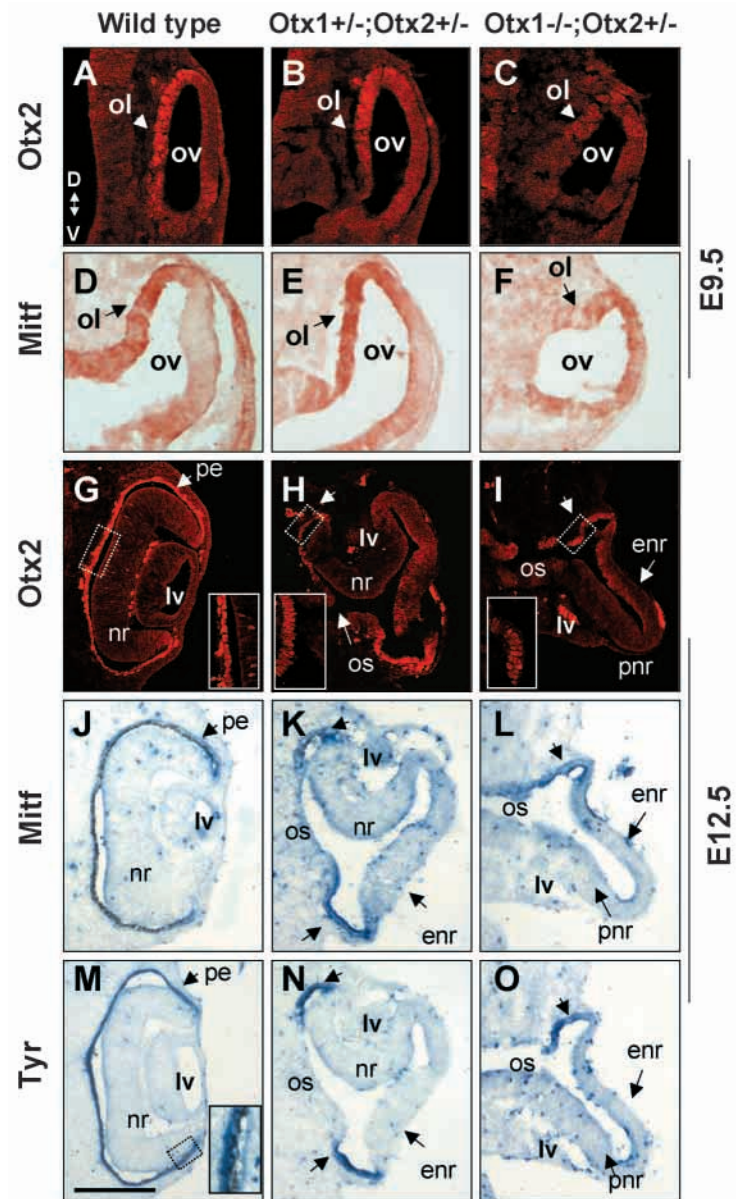


Fig. 4. Localisation of RPE markers in *Otx*-deficient mice. Consecutive sections through E9.5 OV (A-F) or E12.5 OC (G-O) of wild-type (A,D,G,J,M), *Otx1*^{+/-}; *Otx2*^{+/-} (B,E,H,K,N), and *Otx1*^{-/-}; *Otx2*^{+/-} mice (C,F,I,L,O) were immunostained with an antiserum against OTX2 (A-C,G-I) and MITF (D-F) or hybridised with digoxigenin-labelled probes for *Mitf* (J-L) and *tyrosinase* (*Tyr*; M-O). Note how in mutant mice the abnormal distribution of MITF extends through the OV (D-F) overlapping with the ventrally diffused expression of OTX2 (A-C). Note the abnormal folding of the OC in *Otx1*^{+/-}; *Otx2*^{+/-} and *Otx1*^{-/-}; *Otx2*^{+/-} mutants (J compared with K,L). The outer layer of the OC is thicker (inset in G-I) and lacks pigmentation and expression of *Mitf* (K,L) and *tyrosinase* (*Tyr*; N,O). Note that in mutant mice small patches of RPE co-express OTX2, *Mitf* and *tyrosinase* (arrows in H,I,K,L,N,O). enr, ectopic neural retina; lv, lens vesicle; nr, neural retina; ol, outer layer; os, optic stalk; ov, optic vesicle; pe, retina pigment epithelium; pnr, presumptive neural retina. Bar: 200 μm (A-F), 300 μm (G-O).

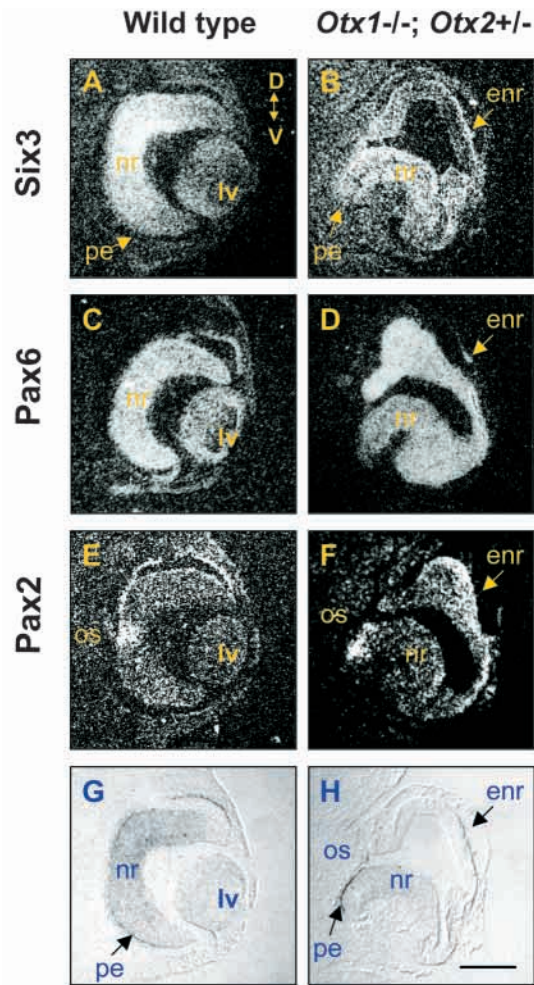


Fig. 5. Gene patterning in the OC of E12.5 *Otx*-deficient mice. The expression domains of *Six3* (A,B), *Pax6* (C,D) and *Pax2* (E,F) in wild type (A,C,E,G) and *Otx1*^{-/-}; *Otx2*^{+/-} (B,D,F,H) mice are illustrated with dark-field views of frontal paraffin sections hybridised with radioactive probes (A-F). Corresponding cytoarchitecture is shown with DIC images in G and H. Note the 'ectopic' expression of *Pax6* and *Six3* in the outer layer of the OC (B,D) and the extension of the *Pax2* domain (F) in *Otx1*^{-/-}; *Otx2*^{+/-} embryos. enr, ectopic neural retina; lv, lens vesicle; nr, neural retina; os, optic stalk; pe, retina pigment epithelium. Bar, 200 μ m.

as well as in the OV (Fig. 1; Simeone et al., 1993; Bovolenta et al., 1997; Zygar et al., 1998). In *Otx1*^{-/-}; *Otx2*^{+/-} mice, lens development was clearly abnormal, as better appreciated by the detection of the expression of α -A-crystallin and *Prox1* (Fig. 7), two lens specific markers (Oliver et al., 1993; Oguni et al., 1994). At E10.5 the lens pit was formed in wild-type embryos and the elongated ectodermal cells expressed α -A-crystallin (Fig. 7A). In contrast, in *Otx1*^{+/-}; *Otx2*^{+/-} and *Otx1*^{-/-}; *Otx2*^{+/-} embryos the lens vesicle was either poorly invaginated, expressing low levels of α -A-crystallin, or remained as an ectodermic α -A-crystallin-negative placode (Fig. 7B,C). From E12.5 onward, when the lens vesicle is formed, all the wild type and *Otx1*^{+/-}; *Otx2*^{+/-} and 88% of the *Otx1*^{-/-}; *Otx2*^{+/-} mice presented lens tissue, expressing both α -A-crystallin and *Prox1*. In the mutants, lens tissue was however reduced (Fig. 7D-I). These results suggest that a delay or a failure in the

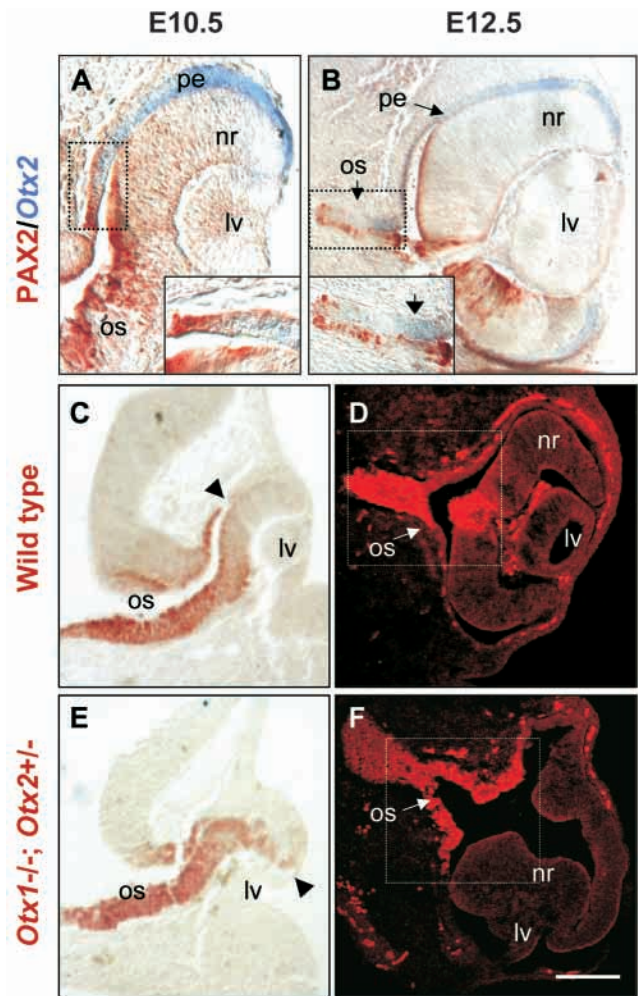


Fig. 6. PAX2 localisation in the developing eye. Frontal sections of wild-type (A-D) and *Otx1*^{-/-}; *Otx2*^{+/-} (E-F) mice at E10.5 (A,C,E) and E12.5 (B,D,F) were immunostained with antiserum against PAX2. *Otx2* expression was localised in wild-type embryos (A,B) with a digoxigenin-labelled probe. Note that *Otx2* and PAX2 colocalise only in a few cells at the boundary of the two expression domains (inset in A, B). In *Otx1*^{-/-}; *Otx2*^{+/-} mice PAX2 expression is extended into the neural NR (compare C and E) and the RPE (D,F) territories. lv, lens vesicle; nr, neural retina; os, optic stalk. Bar: 150 μ m (A,C,E), and 200 μ m (B,D,F).

progression of lens development could account for its absence or reduced size observed in *Otx*-deficient mice.

Improperly patterned RPE differentiates into a NR-like structure in *Otx1*^{-/-}; *Otx2*^{+/-} mice

We next asked whether in mutant mice the outer layer of the OC could differentiate according to its new identity of 'prospective NR'. In wild-type embryos, many tubulin β 3 and Islet1-positive RGC and amacrine cells have exited the cell cycle and have migrated to their proper layer at E17.5 (Fig. 8A,G; Young, 1985; Austin et al., 1995). Staining of equivalent sections of E17.5 *Otx1*^{-/-}; *Otx2*^{+/-} embryos demonstrated that tubulin β 3- and Islet1-positive cells accumulated not only on the vitreal surface of the folded mutant NR, but also on the sclera surface of the 'ectopic' NR (Fig. 8B,C,H,I). In addition, these ectopically differentiated neurones appear to extend

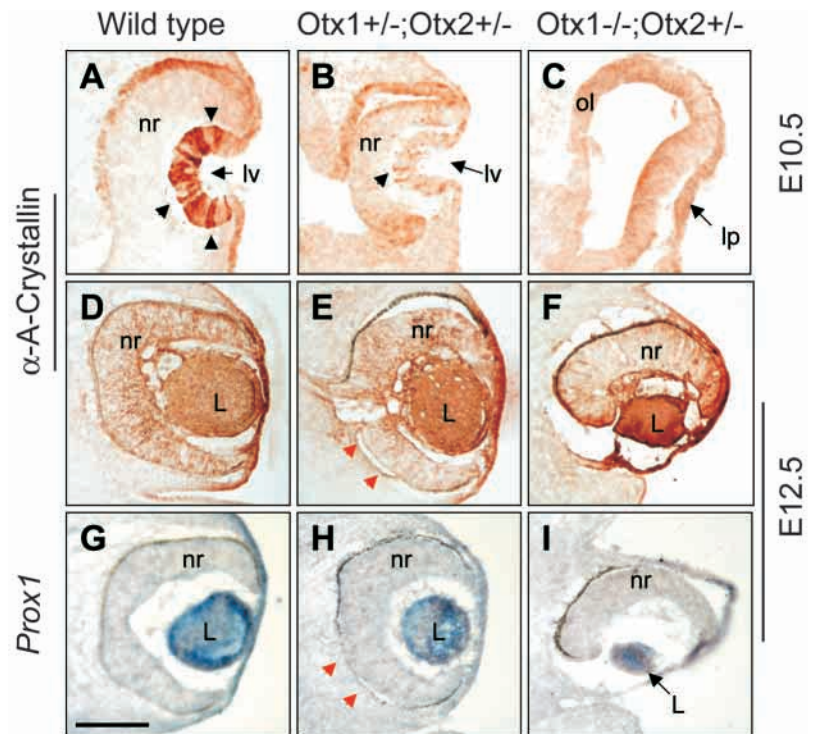


Fig. 7. Lens development abnormalities in *Otx*-deficient mice. Adjacent sections through E10.5 (A-C) and E12.5 OC (D-I) of wild-type (A,D,G), *Otx1*^{+/-}; *Otx2*^{+/-} (B,E,H), and *Otx1*^{-/-}; *Otx2*^{+/-} (C,F,I) mice were immunostained with a polyclonal antiserum against α -A-crystallin (A-F) or hybridised with digoxigenin-labelled probe for *Prox1* (G-I). Note that lens development is delayed and the overall size of the lens is reduced in *Otx1*^{-/-}; *Otx2*^{+/-} embryos (C,F,I). Lack of RPE differentiation is evident in *Otx1*^{+/-}; *Otx2*^{+/-} mice even in the presence of an apparently normal lens (arrowheads in E,H). L, lens; lp, lens placode; nr, neural retina; ol, outer layer. Bar: 200 μ m (A-C), 300 μ m (D-I).

neurofilament- (not shown) and tubulin β 3-positive fibres, which accumulated in aberrant positions at the sclera surface of the eye (Fig. 8B,C). This inverted configuration of the 'newly generated retina' was confirmed by staining with anti-Ph-H3, a mitotic marker (Fig. 8D-F). As a general rule in the CNS, neuroepithelial cells undergo cell division in the ventricular layer, whereas differentiating neurones migrate towards the pial surface (Sauer, 1935; Jacobson, 1968). Therefore, the mirror image organisation of the two 'neural retinas' in the mutant mice reflects the inverted orientation of the outer in relation to the inner neuroepithelial layer of the OC.

NR development is impaired in *Otx1*^{-/-}; *Otx2*^{+/-} mice

In a second wave of expression, both *Otx1* and *Otx2* are expressed in the NR when its differentiation begins (Fig. 1; Simeone et al., 1993; Bovolenta et al., 1997). In particular, *Otx2* expression was observed in postmitotic neuroblasts committed to both neuronal and the glia cell types (Bovolenta et al., 1997). We therefore asked whether retina neurogenesis was affected in *Otx1*^{-/-}; *Otx2*^{+/-} mice.

In all developing structures, neurogenesis is controlled by the balance between cell proliferation, differentiation and apoptosis. In retinas from E18.5 wild-type embryos, Ph-H3-positive mitotic cells were located, as expected, in the ventricular layer (Fig. 9A). In the corresponding retina sections of mutant mice the number of P-H3-positive cells appeared greatly increased throughout the folded NR (Fig. 9B,C). Apoptotic cell death, identified by Cresyl Violet staining (Sengelaub and Finlay, 1982), was not a prominent feature in wild-type retina at this stage but was strikingly frequent in both the RGC and inner nuclear layers of the mutants (Fig. 9D-F). In mice, RGC and amacrine cells represent the majority of differentiated cells in E18.5 retinas (Young, 1985). These cell types, labelled by antibodies against islet-1 were quite

abundant in their respective layers in wild-type embryos (Fig. 9G), while a clear decrease in positive cells was evident in mutant retinas (Fig. 9H-I). These defects were detected in 25% of the *Otx1*^{+/-}; *Otx2*^{+/-} and in all the *Otx1*^{-/-}; *Otx2*^{+/-} animals analysed at the stages from E17.5 to P1.

Statistical analysis of the data described above was performed on equivalent retinal sections from wild-type and *Otx1*^{-/-}; *Otx2*^{+/-} mice ($n=4$). As clearly shown in Fig. 9C,F,I, in the presence of a single *Otx2* allele, retina cell proliferation and cell death were dramatically increased, while cell differentiation was severely impaired. An easy interpretation of these data is however obscured by the indications that NR folding and stratification depends on the presence of the lens (Ashrey-Padan et al., 2000; Yamamoto and Jeffery, 2000) while its differentiation and survival relies on signals generated from the RPE (Raymond and Jackson, 1995; Bovolenta et al., 1996; Frade et al., 1996). Therefore, the precise role of OTX proteins during NR differentiation needs to be established in a different experimental model where lens and RPE will be not affected.

DISCUSSION

Our analysis of the ocular phenotype of mice carrying different functional copies of *Otx1* and *Otx2* provide evidences that a minimal dose of *Otx* genes is required for the proper development of the eye. *Otx* genes are necessary for OV folding and for the establishment of the RPE identity. In the presence of a minimal level of OTX protein, the RPE-specific genes *Mitf* and *tyrosinase* are not activated in the outer layer of the OC. Instead, this region will go on to differentiate as an 'ectopic' NR. Additional defects include the alterations in size and position of the lens and an abnormal rate of cell proliferation, differentiation and death in the NR proper.

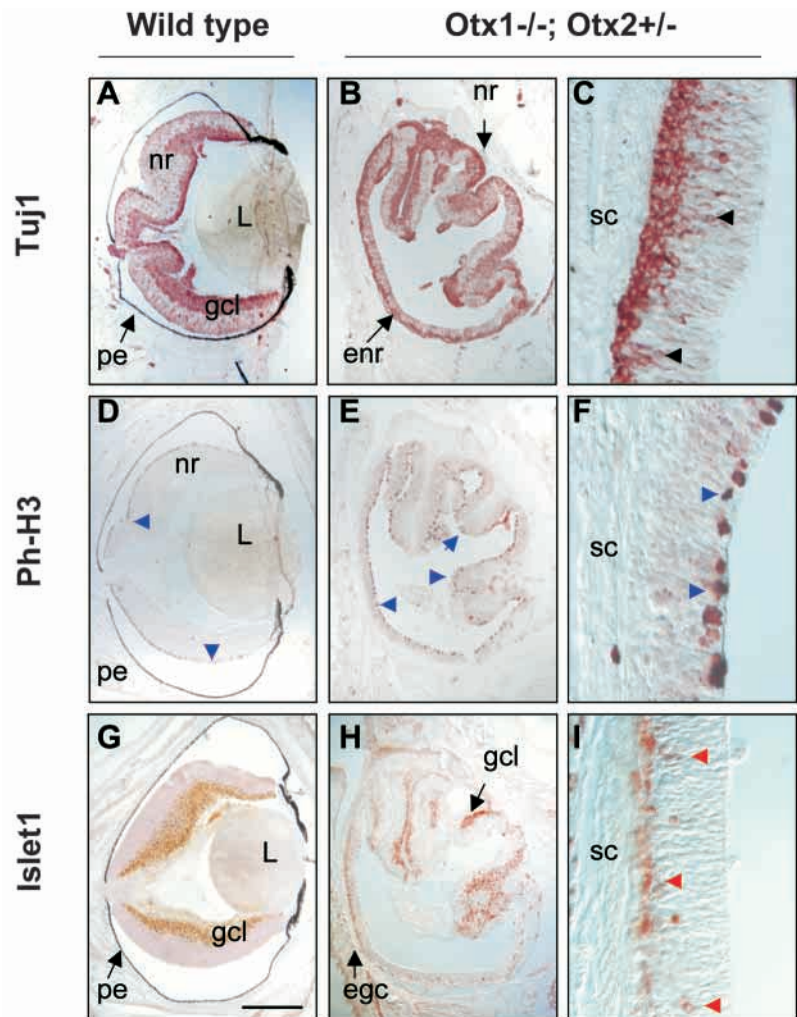
Fig. 8. Improperly patterned RPE differentiates into a NR-like structure in *Otx1*^{-/-}; *Otx2*^{+/-} mice. Paraffin sections at equivalent axial levels through the eye of E17.5 wild type (A,D,G) and *Otx1*^{-/-}; *Otx2*^{+/-} mice (B,C,E,F,H,I). C,F and I are high power views of the ectopic neural retinas illustrated in B,E and H, respectively. Sections were immunostained with antiserum against Tuj1 (A-C), Ph-H3 (D-F), and Islet1 (G-I). Note the presence of Tuj1- and Islet1-positive differentiated neurons not only in the vitreal surface of the NR of both wild-type and mutant embryos but also on the sclera side of the 'ectopic' NR (arrowheads in C and I). Mitotic Ph-H3-labeled nuclei are located at the vitreal surface of the 'ectopic' retina (arrowheads in F). egc, ectopic ganglion cells; enr, ectopic neural retina; gcl, ganglion cell layer; L, lens; nr, neural retina; pe, retinal pigment epithelium; sc, sclera. Bar: 400 μ m but C,F,I, 250 μ m.

Otx genes are required for eye development in a dose dependent manner

We have shown here that eye development in the mouse depends on the number of functional copies of *Otx*. Thus, 100% of the embryos carrying the minimum *Otx* dosage compatible with viability (*Otx1*^{-/-}; *Otx2*^{+/-}) presented gross eye malformations, whereas only 30% of the double heterozygous (*Otx1*^{+/-}; *Otx2*^{+/-}) embryos showed similar phenotypes. *Otx* genes cooperate in the specification of the mesencephalic-metencephalic territories and in the development of the inner ear. However, the threshold of OTX proteins required for proper eye development appears to be higher than those essential for the specification of these regions, given that no inner ear or brain defects were found in double (*Otx1*^{+/-}; *Otx2*^{+/-}) heterozygous embryos (Acampora et al., 1997; Suda et al., 1997; Morsli et al., 1999). In addition, our data indicate that eye development depends on the identity of the functional *Otx* copies present in the mice. Thus, early gross eye malformations, including lens defects, are present in *Otx1*^{+/-}; *Otx2*^{+/-} but not in *Otx1*^{-/-} embryos. In both cases, only two *Otx* alleles are functional but the lack of one of the *Otx2* alleles seems more relevant to eye patterning than that of *Otx1*. In agreement with this, about 70% of the *Otx2* heterozygous mice, generated on a different genetic background from those analysed in this study, presented microphthalmia or anophthalmia and agenesis of the lens (Matsuo et al., 1995). In the case of the lens, the preponderant role of *Otx2* activity is further sustained by the observation that in *Xenopus*, *Otx2* expression in the presumptive lens ectoderm is a prerequisite for the expression of *Pax6* and *Sox*, genes fundamental for lens determination (Zygar et al., 1998). However, and independently of the relative importance of *Otx1* or *Otx2*, it is unclear whether lens defects in *Otx1*^{-/-}; *Otx2*^{+/-} are a totally autonomous process or occur as a secondary event due to the improper patterning of the neuroepithelium, a potential source of lens-inductive signals (Karkinnen-Jaaskelainen, 1978; Porter et al., 1997; Furuta and Hogan, 1998).

Otx functions throughout the different stages of eye development

Evidence from several sources support the idea that *Otx* genes

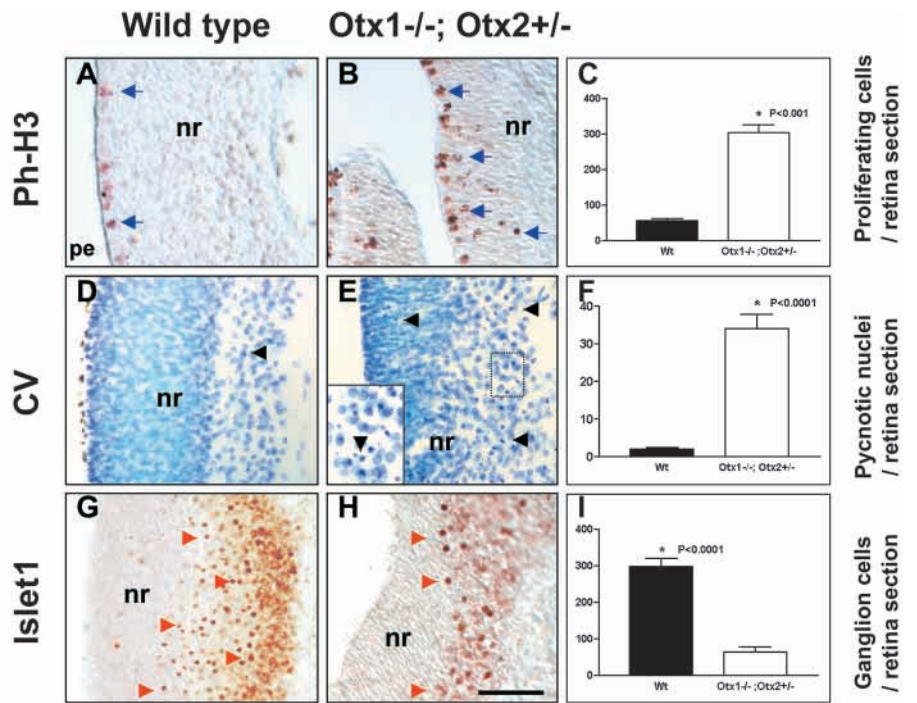


might be involved at different steps of vertebrate eye formation, including the determination of a field permissive for eye formation, the morphogenesis of the lens and OC, and the generation of retina-specific neurones (Bovolenta et al., 1997; Simeone, 1998; Acampora and Simeone, 1999; Chow et al., 1999; Loosli et al., 1999; this study).

In all bilaterian species so far examined, the formation of the anterior neuroectoderm and, therefore, that of the eye field, which lies within the *Otx* expression domain (Finkelstein et al., 1990; Simeone et al., 1992; Li et al., 1994; Loosli et al., 1998; Bruce and Shankland, 1998; Mitsunaga-Nakatsubo et al., 1998; Stornaiuolo et al., 1998; Wada and Saiga, 1999), depends upon *Otx2* function (Acampora et al., 1995). Only in this *Otx2*-positive anterior neuroectoderm, can the overexpression of *Pax6* and *Six3* induce the formation of ectopic eyes in vertebrates (Chow et al., 1999; Loosli et al., 1999), indicating that *Otx* activity provides the anterior neuroectoderm with the necessary competence for ocular specification. We show here that, despite of the subsequent gross eye malformations observed in *Otx1*^{-/-}; *Otx2*^{+/-} mice, OV outpocketing occurs normally, suggesting that a single copy of *Otx2* is sufficient to establish the eye morphogenetic field and to allow its evagination.

Subsequent to eye field specification, the morphogenesis of the vertebrate eye takes place by the infolding of the

Fig. 9. Neural retina development in *Otx1*^{-/-}; *Otx2*^{+/-} mice. Adjacent paraffin sections of E18.5 wild-type (A,D,G) and *Otx1*^{-/-}; *Otx2*^{+/-} (B,E,H) retinas were immunostained with antiserum against Ph-H3 (A,B), antibodies against Islet1 (G,H) or stained with Cresyl Violet to determine the amount of apoptosis in the sections (D,E). Note that the number of mitotic (arrows in B compared with A) and apoptotic (arrowheads in E compared with D) cells is greatly increased while those of differentiated cells (compare arrowheads in G and H) is decreased in mutant retinas. Statistical analysis of the number of mitotic, apoptotic and Islet1-positive cells is presented in C,F and I, respectively (*n*=4). Note that in all cases there is significant difference between control and *Otx*-deficient mice. enr, ectopic neural retina; gcl, ganglion cell layer; nr, neural retina; pe, retinal pigment epithelium. Bar, 100 μm.

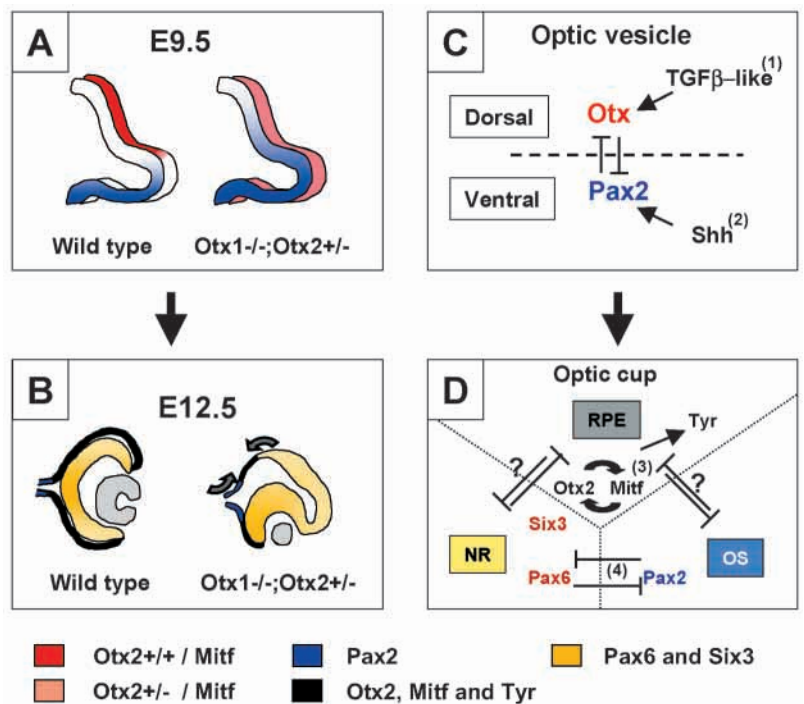


undifferentiated eye primordia into a bi-layered cup. In this structure, both the basic architecture of the adult organ and the identity of the different tissues become established by the coordinated action of autonomously expressed transcription factors and inductive signals. In the presence of low levels of *Otx* activity, folding of the OV is severely affected. In *Otx* mutants, the delayed or abnormal development of the lens placode and/or the low levels of OTX protein in the lens ectoderm (Hyer et al., 1998; Nguyen and Arnheiter, 2000) could be in part responsible for the morphological defect of the OV. Similar abnormal OV invagination has indeed been observed in a lens-specific *Pax6* conditional mouse mutation, where the absence of the lens placode has permitted the analysis of its effects on vesicle morphogenesis (Ashrey-Padan et al., 2000). Alternatively, OTX protein activity could be directly implicated in the control of morphogenetic movements of the cells. Association between cell migration and *Otx2* expression has been proposed in the mouse olfactory system (Mallamaci et al., 1996) and gastrulation movements are severely affected in *Otx2*^{-/-} mice (Simeone, 1998). Furthermore, *Otx2* appears to control the

expression of molecules implicated in cytoskeleton organisation and cell-cell interaction, such as calponins, R-cadherin or ephrinA2 (Rhinn et al., 1999; Morgan et al., 1999) and the ectopic expression of *Otx1* and *otd* results in strong cell aggregation in zebrafish (Bellipanni et al., 2000). Therefore, it is possible that alterations of similar molecular pathways are responsible for aberrant morphogenesis of the OC in *Otx1*^{-/-}; *Otx2*^{+/-} and *Otx1*^{+/-}; *Otx2*^{+/-} embryos.

In addition to morphogenetic defects, *Otx*-deficient mice show altered expression of genes involved in the proper subdivision of the OV territory into RPE, NR and OS. Soluble

Fig. 10. Proposed model for gene interactions involved in the establishment of the territories of the vertebrate eye. (A,B) Schematic summaries of the distribution of eye patterning genes in wild-type and *Otx1*^{-/-}; *Otx2*^{+/-} mice at E9.5 and E12.5. Panels C and D suggest how genes necessary to confer the identity of one territory (i.e. Otx with the PE) may contribute to restrict the expression of genes necessary for the specification of the nearby regions (OS and NR). This model is based on the present findings and on the results reported in (1) Fuhrmann, 2000; (2) McDonald et al., 1995; (3) Nguyen and Arnheiter, 2000; (4) Schwarz et al., 2000 (see Discussion for details).



molecules derived from adjacent tissues (the surface ectoderm and the surrounding mesenchyme) appear to influence NR versus RPE fate decision. Fibroblast growth factors, normally expressed in the PLE, can determine neural retina identity even in tissue fated to give rise to RPE (Pittack et al., 1991; Pittack et al., 1997; Guillemot and Cepko, 1992; Hyer et al., 1998; Zhao and Overbeek, 1999; Nguyen and Arnheiter, 2000). In contrast, extraocular mesenchyme is the source of molecules, possibly activin-like signals, capable of repressing NR while activating RPE markers (Fuhrmann et al., 2000). The regulation of tissue-specific genes might therefore be the results of these (positive and negative) inductive signals. However, the suppression of PLE development does not affect the formation and the separation of NR and RPE progenitor cell (Ashery-Padan et al., 2000), giving strong support to our finding that the loss of functional alleles in *Otx* mutants is the direct cause of the failure of RPE determination. Interestingly, in *Otx* mutants the expression of *Mitf* and *tyrosinase* is also largely absent and maintained only in little patches of tissue where OTX2 is also localised. In *Mitf* mutants the expression of *Otx2* is specifically down regulated in those areas where RPE has not differentiated (Nguyen and Arnheiter, 2000). This suggests that both transcription factors cooperate to determine the identity and possibly maintain the function of the RPE. However, we believe that *Otx* genes have a prevalent role in this process, because the expression of *Otx* precedes that of *Mitf* (Nguyen and Arnheiter, 2000). In addition, in most cases the entire RPE territory is affected in *Otx*-deficient mice, whereas only patches of the dorsal RPE is affected when the entire *Mitf* gene is lost (*Mitf^{vga-9/vga-9}*; Nguyen and Arnheiter, 2000).

The enlargement of the expression domains of OS- (*Pax2*) and NR- (*Pax6* and *Six3*) specific genes further suggests the importance of *Otx* activity in the proper subdivision of the OV. Whether OTX proteins repress the expression of *Pax2*, *Six3* or *Pax6* directly or indirectly needs to be determined. However, a recent report shows that the identity of the OS and OC depends on the specific expression and reciprocal transcriptional repression of *Pax2* and *Pax6* (Schwarz et al., 2000). Similar mechanisms may be acting in the segregation of other genes involved in the establishment of RPE/OS and RPE/NR boundaries. A hypothetical model that takes into account all the data described above is schematically illustrated in Fig. 10. In this simplified scheme, the expression, regulated respectively by TGF β -like and *Shh* signalling (McDonald et al., 1995), and the reciprocal repression of *Otx* and *Pax2* will determine, in coordination with other genes as, for instance, *Vax1* and *Vax2* (Barbieri et al., 1999; Bertuzzi et al., 1999; Hallonet et al., 1999), the initial dorsal and ventral patterning of the OV (Fig. 10A,C). In this respect, we show that initially only the expression of *Pax2*, but not that of either *Six3* or *Pax6* is significantly altered in *Otx*-deficient mice. In addition, in *Pax2* null mice, pigmented cells invade the optic stalk (Torres et al., 1996). In support of a possible antagonistic interaction between *Otx* and *Pax2*, it is also worth mentioning that the two genes may be acting in a similar antagonistic manner to establish the pattern of the inner ear primordium. *Otx* and *Pax2* genes are expressed in the otic vesicle in the presumptive vestibular and cochlear domains, respectively (Morsli et al., 1999; Hidalgo-Sanchez et al., 2000). Furthermore, *Pax2*^{-/-} and *Otx* mutants (*Otx1*^{-/-}, and *Otx1*^{-/-}; *Otx2*^{+/-}) show divergent

otic phenotypes, lacking the auditory (cochlea and spiral ganglion) and the vestibular (semicircular canal, lateral ampulla, utriculosaccular and cochleosaccular duct) parts of the organ, respectively (Torres et al., 1996; Morsli et al., 1999).

Later, as the RPE, NR and OS progenitor cells acquire their identity, the mutual positive interaction between *Otx* and *Mitf* in the dorsal portion of the eye will induce and probably maintain the network of genes necessary for the establishment RPE identity (Fig. 10B,D; Bentley et al., 1994). Complete segregation of eye territories would also imply the mutual regulation of different OS, NR and RPE specific genes. Thus, regulation of *Otx2* and *Pax2* expression in cells located at the RPE/OS border will finally establish the separation of the two territories with a mechanism similar to that proposed for *Pax2* and *Pax6* interaction at the OS/OC boundary (Schwarz et al., 2000). In *Otx* mutants, *Pax6* expression appeared unaffected in spite of the expansion of the *Pax2*-positive territory. Whether this is due to an independent autoregulation of *Pax6* expression (Schwarz et al., 2000) or whether OTX proteins could also contribute to the direct regulation of *Pax6* expression is not clear. Indeed, the precise hierarchical interactions among all the genes required for the OV subdivision are just beginning to be elucidated.

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