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Ectopic lens induction in fish in response to the murine homeobox gene *Six3*

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Abstract

Recent findings show an unexpected conservation of genes involved in vertebrate and insect eye development. The *Drosophila* homeobox gene *sine oculis* is crucial for eye development. Its murine homologue, *Six3* is expressed in the anterior neural plate, a region which is involved in lens induction in *Xenopus*. To examine whether *Six3* participates in the process of eye formation, mouse *Six3* was ectopically expressed in fish embryos. The results show that *Six3* is sufficient to promote ectopic lens formation in the area of the otic vesicle and that retinal tissue is not a prerequisite for ectopic lens differentiation. Our findings suggest a conserved function for *Six3* in metazoan eye development.

Keywords: Lens formation; *Six3*; Homeobox gene; Placodes; Medaka fish

1. Introduction

Several genes thought to be involved in eye development have recently been isolated in both *Drosophila* and vertebrates. The paired and homeobox-containing gene *Pax6* has been shown to play a crucial role in this process in mammals and insects (Hogan et al., 1986; Hill et al., 1991; Walther and Gruss, 1991; Quiring et al., 1994; Halder et al., 1995a; Halder et al., 1995b). This led to the hypothesis that vertebrate and insect eye development may share an evolutionary conserved pathway (Quiring et al., 1994; Halder et al., 1995b). Thus, it seems likely that other structurally and functionally conserved genes involved in vertebrate and *Drosophila* eye development will be identified.

The *Drosophila* homeobox gene *sine oculis* is required for the development of the visual system (Cheyette et al., 1994). Its murine homologue *Six3*, is expressed in the anterior neural plate, the optic vesicles and the lens (Oliver et al., 1995). This expression pattern suggests that *Six3* is also involved in eye development. Vertebrate lens formation involves a multistep process, starting at the gastrula stage, and requires an inductive signal emanating from the anterior neural plate (Jacobson, 1966; Henry and Grainger, 1987; Grainger et al., 1988; Saha et al., 1989; Grainger, 1992). This signal elicits a lens-forming bias in the lens competence region of the head ectoderm. In mouse, *Six3* is expressed in the anterior neural plate at the time when this inductive signal is thought to act (Oliver et al., 1995). Later on, the optic vesicle appears to play a key role in lens differentiation (Grainger, 1992).

2. Results and discussion

To examine whether *Six3* participates in the process of eye formation, mouse *Six3* was ectopically expressed in the killifish medaka (*Oryzias latipes*; Yamamoto, 1975).

Plasmid DNA containing the mouse *Six3* cDNA under the control of the cytomegalovirus (CMV) promoter/enhancer (pC5*Six3*) was injected into 2–4-cell stage medaka embryos for transient mosaic expression of the *Six3* transgene starting at mid-blastula transition (Stuart et al., 1990; Winkler et al., 1991). To analyze the distribution of the pC5*Six3*-expressing cells, whole mount in situ hybridization was performed at different time points after injection. As shown in Fig. 1, transgene-expressing cells are randomly distributed in the injected embryo allowing a

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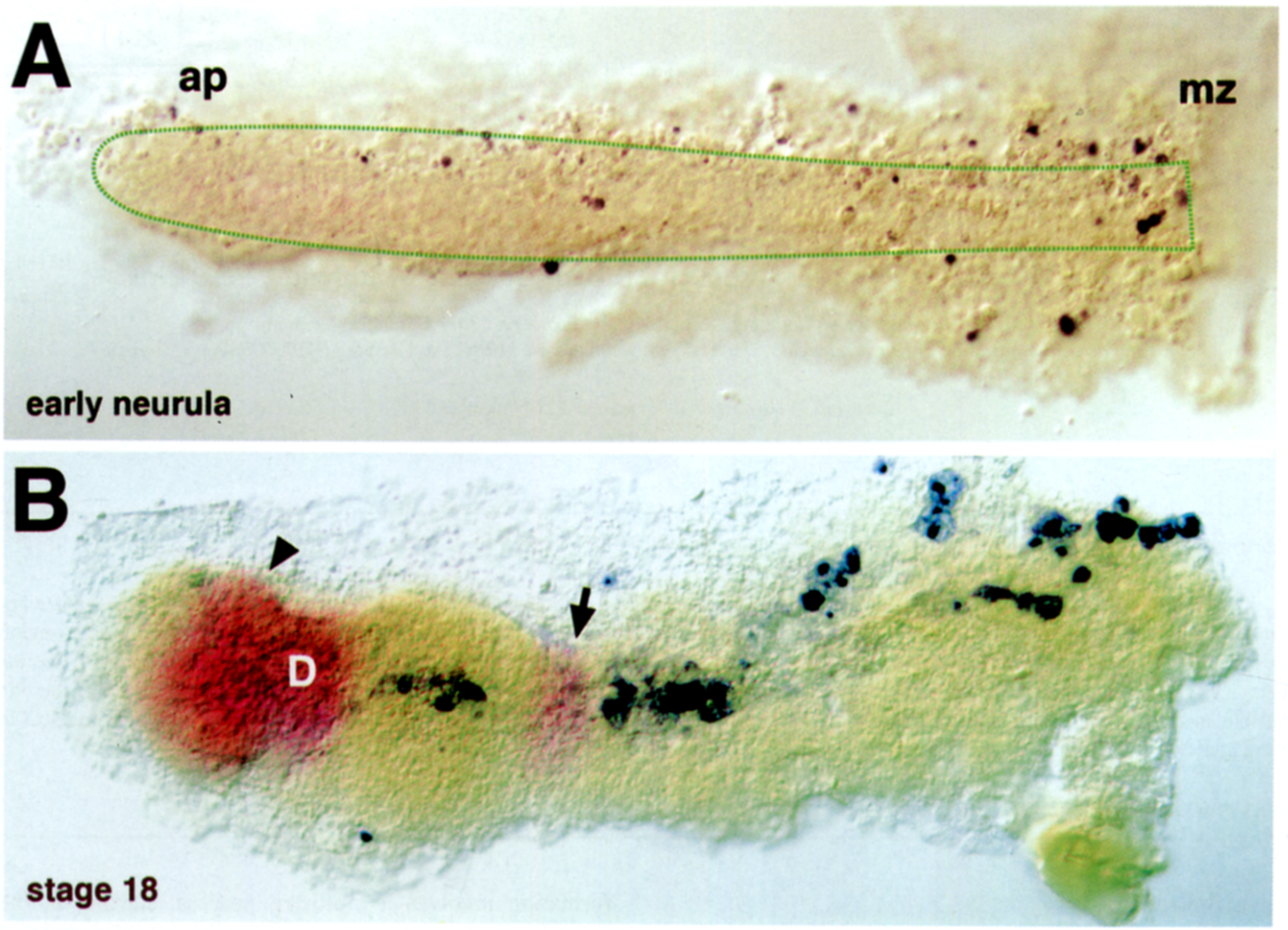

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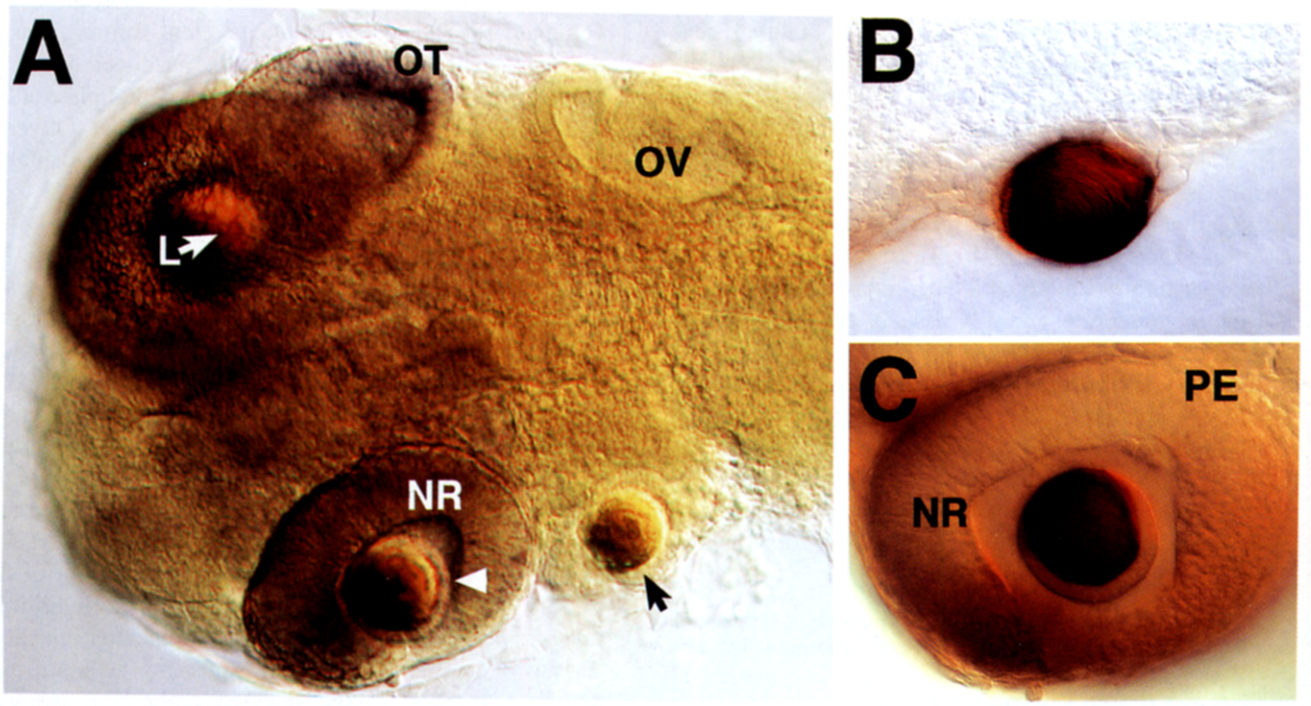


clonal analysis of the possible effects in different regions of the developing embryo.

In wild type medaka, the first visible sign of lens formation is a thickening of the lens placode at 30 h of development. Characteristic onion-like, concentric arrangement of the lens fiber cells surrounded by the lens epithelium is observed at day 3 of development (late organogenesis stage) (Fig. 2C) (Iwamatsu, 1994). In 2.5% of the embryos injected with pC5*Six3* plasmid DNA ($n = 15/609$) an ectopic lens was observed at day 3 (Fig. 2A,B). In all cases, only one ectopic lens was formed, being slightly smaller than the wild type lens (Fig. 2A). The ectopic lenses exhibit the characteristic lens morphology (Figs. 2B,C and 4A,D). Furthermore, γ-crystallin was immunodetected in the ectopic lenses with a polyclonal antiserum raised against *Xenopus* lens proteins, unambiguously identifying the ectopic structures as lenses (Fig. 2B,C).

Although mouse *Six3*-expressing cells are randomly distributed in the embryo at early neurula stages (Fig. 1), ectopic lenses were formed exclusively in place of the otic vesicle (Fig. 2A), which in the wild type embryo will give rise to the inner ear, or in a position close to it (Fig. 4B). This indicates that for the formation of an ectopic lens, a competent tissue (otic placode territory) has to be targeted by *Six3*-expressing cells. *Six3* expression in a non-competent region is not sufficient for ectopic lens formation. Considering the random distribution of the *Six3*-expressing cells and the limited size of the competent tissue, it is to be expected that only in a small percentage of the injected embryos an ectopic lens can form.

As a control, 344 embryos were injected under identical conditions with pCMV5 plasmid DNA lacking the *Six3* cDNA. Formation of ectopic lenses was not observed. The finding that ectopic lenses were observed only in pC5*Six3*-injected embryos indicates that ectopic lens formation depends on ectopic mouse *Six3* expression and that this gene plays a key role in the process of lens formation in vertebrates.

It has been proposed that the retina is required for vertebrate lens differentiation (Grainger et al., 1988; Saha et al., 1989; Henry and Grainger, 1990; Grainger, 1992). Judged on the basis of pigmentation and morphological criteria, none of the injected embryos developed either an ectopic optic cup or a retina neighboring the ectopic lens (Figs. 2–4). The absence of an optic vesicle or a neuroretina in the vicinity of the ectopic lenses was confirmed by the use of medaka *tailless* and *Pax6* expression as molecular markers (Köster, Loosli and Wittbrodt, unpublished results) (Figs. 2–4). In the wild type medaka embryo, *tailless* is expressed as in the mouse (Monaghan et al., 1995) in the developing optic vesicle (not shown) and at later stages in the neuroretina and optic tectum (Fig. 3A). Initially, *Pax6* is expressed in the anterior head ectoderm, the optic vesicles (arrowhead in Fig. 1B) and in the developing central nervous system. Later on, transcripts are detectable in the neuroretina and in the lens epithelium (Fig. 4A,C). Both *tailless* (Figs. 2A and 3B,C) and *Pax6* (Fig. 4B,D) expression were not observed in the vicinity of the ectopic lenses, whereas wild type *tailless* expression was observed in the neuroretina and optic tectum (Fig. 3B), and wild type *Pax6* expression in the neuroretina (not shown) and hindbrain (Fig. 4B). Taken together, these observations provide evidence that neither an optic cup nor a neuroretina are present in the vicinity of the ectopic lenses. This indicates that the presence of retinal tissue is not a prerequisite for the final steps of ectopic lens differentiation.

In murine loss of function *Pax6* mutants the lens is not formed, indicating a key role for *Pax6* in lens formation (Hill et al., 1991; Quinn et al., 1996). We therefore investigated whether ectopic mouse *Six3* expression results in the activation of ectopic *Pax6* expression. Using a double labeling whole mount in situ analysis, the potential interaction of both genes was analyzed. No coexpression of mouse *Six3* and *Pax6* was detected in late neurula stage embryos (Fig. 1B).

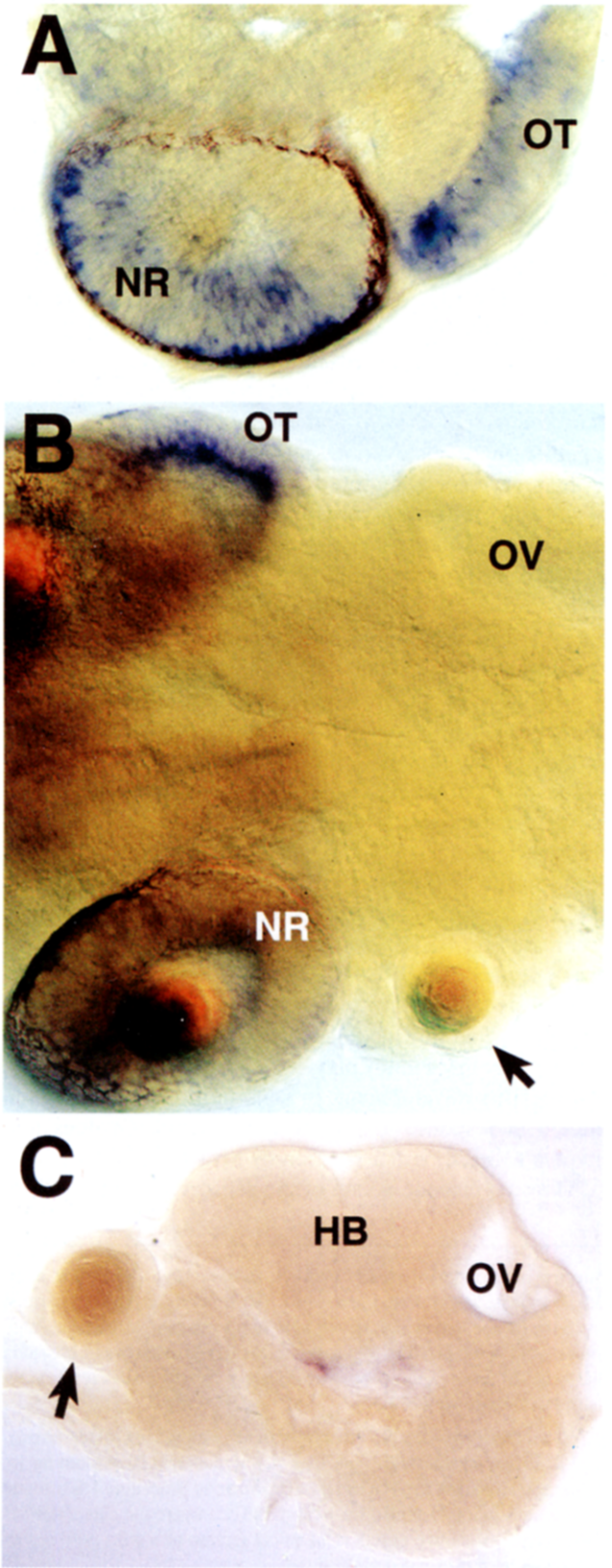
As in the wild type, *Pax6* expression was observed in the lens epithelium of the ectopic lens itself at day 3 (compare Fig. 4C,D). At this stage mouse *Six3* expression does not result in the activation of *Pax6* at ectopic places as indicated by whole mount in situ analysis (arrow in Fig. 4D). Therefore, the observed *Pax6* expression in the ectopic lens epithelium may reflect a late requirement of *Pax6* in lens development. In summary, ectopic lens formation in response to murine *Six3* expression does not involve a direct *Pax6* activation by *Six3*.

The lens placode is one of the sensory placodes which

Fig. 1. Mosaic distribution of *Six3*-expressing clones in injected embryos. (A) Seventy percent epiboly (early neurula, 16 h) embryo showing in dark blue the distribution of the *Six3* expressing clones. The extent of the neural plate is indicated by a dotted green line. (B) Embryo at the late neurula stage (stage 18, 22 h). Red staining corresponds to endogenous *Pax6* expression in the diencephalon (D), developing optic vesicles (arrowhead) and hindbrain area (arrow). Dark blue staining corresponds to the *Six3*-expressing clones. No colocalization of *Pax6* and *Six3* expression was observed. Anterior side of the embryos is oriented to the left. ap, animal pole; mz, marginal zone. In these experiments *Pax6* expression was visualized first to rule out that transgene-expressing cells prevent the detection of potential ectopically *Pax6*-expressing cells.

Fig. 2. Ectopic *Six3* expression promotes lens formation in the region of the otic vesicle. (A) Three day old embryo with an ectopic lens (black arrow) replacing the otic vesicle (OV) on the left side of the embryo. White arrowhead is indicating the wild type lens (L) epithelium. Blue staining in neuroretina (NR) and optic tectum (OT) indicate *tailless* expression after whole mount in situ analysis. The in situ hybridization procedure leads to an unspecific brown staining of the lenses. (B) Transverse vibratome section of an ectopic lens immunostained for γ-crystallin. Compare the characteristic onion-like, concentric arrangement of the lens fiber cells of the ectopic with the wild type lens shown in (C). (C) Optical section of a wild type eye at day 2.5 immunostained for γ-crystallin. PE, pigmented epithelium. Anterior side of the embryo is oriented to the left.

are represented by thickenings of the head ectoderm surrounding the neural plate. The data presented here demonstrate that in addition to the lens placode, the region of the otic placode is competent to form lenses in response to *Six3*, which is in good agreement with previous data obtained by tissue transplantation (Barabanov and Fedtsova, 1982; Saha et al., 1989).



Based on the suggested model for lens induction in the prospective lens ectoderm by the anterior neural plate (Grainger, 1992), we propose that the ectopic *Six3* expression has changed the bias of the otic placode towards the lens pathway by inducing a secreted factor. The finding that mouse *Six3*-expressing cells were detected in the vicinity of several ectopic lenses (arrow in Fig. 4D), whereas no clones were observed in the ectopic lenses proper, hints at a cell non-autonomous process initiated by ectopic *Six3* expression. Consistent with a role of *Six3* in lens induction, in mouse *Six3* is initially expressed in the anterior neural plate, whereas expression in the developing lens starts after lens vesicle formation. The finding that *Pax6* and *tailless* expression are not altered in *Six3*-injected embryos, suggests that *Six3*-promoted ectopic lens formation is independent of a respecification of the hindbrain region towards anterior fates.

The retina has been proposed to be required for lens differentiation (Saha et al., 1989). At the stage at which ectopic lenses are detected, no ectopic retinal tissue was detected in the pC5*Six3*-injected embryos. This suggests that a retina is not required during the final steps of ectopic lens differentiation.

The finding that ectopic expression of a single mouse gene (*Six3*) promotes ectopic lens formation in a fish embryo demonstrates that *Six3* is a key player in vertebrate lens formation. Furthermore, these results suggest that in addition to the conserved role of *Pax6* in vertebrate and insect eye development, the function of additional factors, including *Six3/sine oculis,* are evolutionary conserved in metazoan eye development (Quiring et al., 1994; Zuker, 1994; Halder et al., 1995a; Halder et al., 1995b; Oliver et al., 1995).

3. Experimental procedures

3.1. Fish stocks

Wild type fish were initially purchased from Carolina Biological Supply and kept in a closed stock under natu-

Fig. 3. No retina is present in the vicinity of the ectopic lens. (A–C) In situ analysis of 3 day old embryos using a medaka *tailless* (blue) probe. (A) Transverse vibratome section showing *tailless* staining in neuroretina (NR) and optic tectum (OT). Left half is shown. (B) Ectopic lens surrounded by lens epithelium (arrow) replaces left otic vesicle. Wild type *tailless* expression is detected in the neuroretina (NR) and optic tectum (OT) (compare A), no ectopic staining is found in the vicinity of the ectopic lens. Anterior is to the left. (C) Vibratome section of the embryo shown in (B). Ectopic lens replacing the left otic vesicle and exhibiting the characteristic concentric arrangement of the lens fiber cells and lens epithelium (arrow). No *tailless* expression is detected in the vicinity of the ectopic lens. HB, hindbrain; OV, otic vesicle. Dorsal side is up.

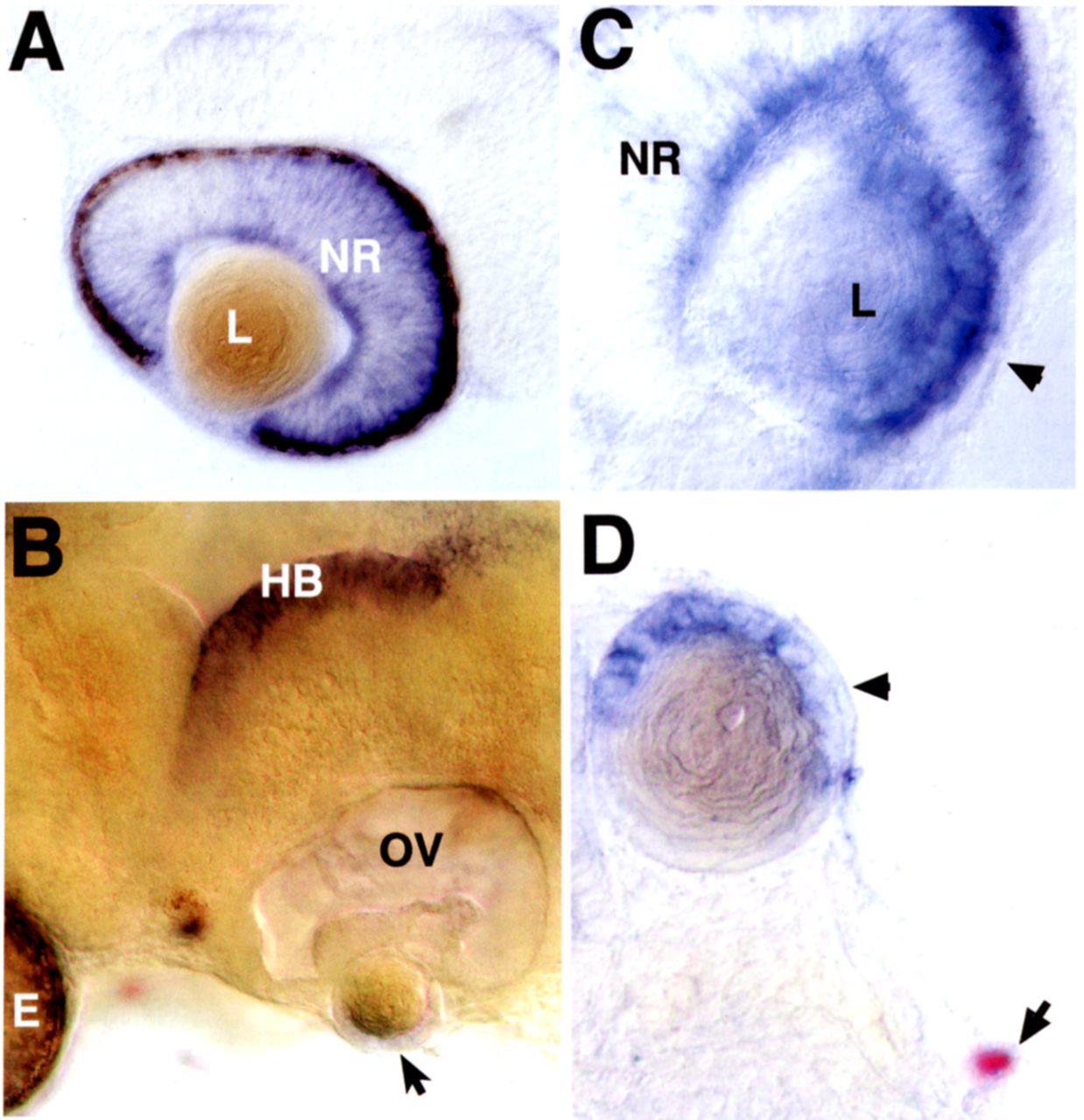


Fig. 4. Ectopic lens exhibits a wild type morphology. (A–D) Whole mount in situ analysis using medaka *Pax6* (blue) and mouse *Six3* (red) probes. (A) Transverse vibratome section of a 3 day old wild type embryo. *Pax6* expression is observed in neuroretina (NR). (B) Three day old embryo with an ectopic lens (arrow) formed in the region abutting the otic vesicle (OV). *Pax6* expression is detected in the eye (E) and hindbrain (HB) but not in the vicinity of the ectopic lens. Anterior is to the left. (C) Transverse vibratome section of a 2 day old embryo showing wild type *Pax6* expression in neuroretina (NR) and lens epithelium (arrowhead). (D) Transverse vibratome section of the embryo shown in (B). Note the characteristic lens morphology. As in the wild type lens (C) *Pax6* expression is detected in the distal portion of the ectopic lens epithelium (arrowhead). Arrow indicates mouse *Six3*-expressing cells (red). No *Pax6* expression is detectable in the vicinity.

ral random breeding conditions according to Yamamoto (1975).

3.2. Cloning

A 1.4 kb EcoRI fragment containing the mouse *Six3* coding region (Oliver et al., 1995) was cloned into pCMV5 (Andersson et al., 1989).

3.3. DNA injections

Qiagen EndoFree purified DNA of pC5*Six3* and

pCMV5 as a control were injected at 100 ng/μl into one blastomere at the 2–4 cell stage and raised as described at 28°C (Stuart et al., 1990; Winkler et al., 1991). As plasmid DNA injected into the fertilized egg does not stably integrate, cell divisions may result in a decrease of the expression level.

In 2.1% of the embryos injected with pC5*Six3* a cyclopia phenotype was found. In the control experiments cyclopes were found in 1.5% of the embryos indicating that this effect is an unspecific result of injection of plasmid DNA at high concentration.

In 3.6% of the embryos injected with pC5*Six3* plasmid DNA a split posterior axis was found. Unspecific effects such as general developmental retardation or a kinked axis were seen in 18%. In control injected embryos a split posterior axis was found in 0.9%; unspecific effects were observed in 14.2%.

3.4. Whole mount in situ hybridization

Double labeling in situ hybridization was performed using digoxigenin and fluoresceine-labeled antisense RNA probes following standard procedures (Hauptmann and Gerster, 1994). In all cases the fast red detection (red) was performed first, followed by the NBT/BCIP staining (blue).

3.5. Immunodetection

Immunodetection of lens γ-crystallins was performed as described (Wittbrodt and Rosa, 1994) using a polyclonal antiserum directed against *Xenopus* lens proteins (Henry and Grainger, 1990) kindly provided by R.M. Grainger, at a dilution of 10^{-3}.

3.6. Vibratome sectioning

Vibratome sections were performed as described (Bober et al., 1994).

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References

Andersson, S., Davis, D.N., Dahlback, H., Jornvall, H. and Russel, D.W. (1989) Cloning, structure and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. J. Biol. Chem. 264, 8222–8229.

Barabanov, V.M. and Fedtsova, N.G. (1982) The distribution of lens differentiation capacity in the head ectoderm of chick embryos. Differentiation 21, 183–190.

Bober, E., Franz, T., Arnold, H.H., Gruss, P. and Tremblay, P. (1994) Pax-3 is required for the development of limb muscles: a possible role for the migration of dermomyotomal muscle progenitor cells. Development 120, 603–612.

Cheyette, B.N.R., Green, P.J., Martin, K., Garren, H., Hartenstein, V. and Zipursky, S.L. (1994) The *Drosophila* sine oculis locus encodes a homeodomain-containing protein required for the development of the entire visual system. Neuron 12, 977–996.

Grainger, R.M. (1992) Embryonic lens induction: shedding light on vertebrate tissue determination. Trends Genet. 8, 349–355.

Grainger, R.M., Henry, J.J. and Henderson, R.A. (1988) Reinvestigation of the role of the optic vesicle in embryonic lens induction. Development 102, 517–526.

Halder, G., Callaerts, P. and Gehring, W.J. (1995a) Induction of ectopic eyes by targeted expression of the eyeless gene in *Drosophila*. Science 267, 1788–1792.

Halder, G., Callaerts, P. and Gehring, W.J. (1995b) New perspectives on eye evolution. Curr. Opin. Gen. Dev. 5, 602–609.

Hauptmann, G. and Gerster, T. (1994) Two-color whole-mount in situ hybridization to vertebrate and *Drosophila* embryos. Trends Genet. 10, 266.

Henry, J.J. and Grainger, R.M. (1987) Inductive interactions in the spatial and temporal restriction of lens-forming potential in embryonic ectoderm of *Xenopus laevis*. Dev. Biol. 124, 200–214.

Henry, J.J. and Grainger, R.M. (1990) Early tissue interactions leading to embryonic lens formation in *Xenopus laevis*. Dev. Biol. 141, 149–163.

Hill, R.E., Favor, J., Hogan, B.L.M., Ton, C.C.T., Saunders, G.F., Hanson, I.M., Prosser, J., Jordan, T., Hastie, N.D. and van Heyningen, V. (1991) Mouse small eye results from mutations in a paired-like homeobox containing gene. Nature 354, 522–525.

Hogan, B.L.M., Horsburgh, G., Cohen, J., Hetherington, C.M., Fisher, G. and Lyon, M.F. (1986) Small eyes (Sey): a homozygous lethal mutation on chromosome 2 which affects the differentiation of both lens and nasal placodes in the mouse. J. Embryol. Exp. Morphol. 97, 95–110.

Iwamatsu, T. (1994) Stages of normal development in the medaka *Oryzias latipes*. Zool. Sci. 11, 825–839.

Jacobson, A.G. (1966) Inductive processes in embryonic development. Science 152, 25–34.

Monaghan, A.P., Grau, E., Bock, D. and Schütz, G. (1995) The mouse homolog of the orphan nuclear receptor tailless is expressed in the developing forebrain. Development 121, 839–853.

Oliver, G., Mailhos, A., Wehr, R., Copeland, N.G., Jenkins, N.A. and Gruss, P. (1995) Six3, a murine homologue of the sine oculis gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. Development 121, 4045–4055.

Quinn, J.C., West, J.D. and Hill, R.E. (1996) Multiple functions for Pax6 in mouse eye and nasal development. Genes Dev. 10, 435–446.

Quiring, R., Walldorf, U., Kloter, U. and Gehring, W.J. (1994) Homology of the eyeless gene of *Drosophila* to the small eye gene in mice and aniridia in humans. Science 265, 785–789.

Saha, M., Spann, C.L. and Grainger, R.M. (1989) Embryonic lens induction: more than meets the optic vesicle. Cell Differ. Dev. 28, 153–172.

Stuart, G.W., Vielkind, J.R., McMurray, J.V. and Westerfield, M. (1990) Stable lines of transgenic zebrafish exhibit reproducible patterns of transgene expression. Development 109, 577–584.

Walther, C. and Gruss, P. (1991) Pax-6, a murine paired box gene, is

expressed in the developing CNS. Development 113, 1435–1449.

Winkler, C., Vielkind, J.R. and Schartl, M. (1991) Transient expression of foreign DNA during embryonic and larval development of the medaka fish (*Oryzias latipes*). Mol. Gen. Genet. 226, 129–140.

Wittbrodt, J. and Rosa, F.M. (1994) Disruption of mesoderm and axis formation in fish by ectopic expression of activin variants: the role of maternal activin. Genes Dev. 8, 1448–1462.

Yamamoto, T. (1975) Medaka (Killifish), Biology and Strains. Keigaku, Tokyo.

Zuker, C.S. (1994) On the evolution of eyes: would you like it simple or compound? Science 265, 742–743.