RESEARCH COMMUNICATION

Six3 overexpression initiates the formation of ectopic retina

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The homeobox gene *sine oculis* (*so*) is essential for visual system formation in *Drosophila*. A vertebrate member of the *so/Six* gene family, *Six3*, is expressed in the developing eye and forebrain. Injection of *Six3* RNA into medaka fish embryos causes ectopic *Pax6* and *Rx2* expression in midbrain and cerebellum, resulting in the formation of ectopic retinal primordia. Injected mouse *Six3* RNA initiates ectopic expression of endogenous medaka *Six3*, uncovering a feedback control of *Six3* expression. Initiation of ectopic retina formation reveals a pivotal role for *Six3* in vertebrate retina development and hints at a conserved regulatory network underlying vertebrate and invertebrate eye development.

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In vertebrate eye development, the anterior ectoderm gives rise to the retinal primordium in the anterior neuroectoderm and the lens primordia in the lateral head ectoderm. The formation of retinal and lens primordia requires a closely coordinated development involving inductive interactions between these structures (Grainger 1996). *Pax6*, a structurally conserved transcription factor is expressed in these primordia and was shown to play a key role in vertebrate and invertebrate eye development (Hill et al. 1991; Ton et al. 1991; Matsuo et al. 1993; Quiring et al. 1994; Halder et al. 1995a). Several other vertebrate and invertebrate genes expressed in the developing eye are also structurally conserved, suggesting an evolutionary conservation of the mechanisms underlying eye development across species lines (Quiring et al. 1994; Halder et al. 1995b; Desplan 1997; Xu et al. 1997). In *Drosophila* the development of the larval and adult visual system requires the activity of the homeobox-containing transcription factor *sine oculis* (*so*) (Cheyette et al. 1994). A family of vertebrate homologs has been isolated from a variety of vertebrate species (Oliver et al. 1995a,b; Kawakami et al. 1996a,b; Bovolenta et al. 1998; Kobayashi et al. 1998; Loosli et al. 1998; Seo et al. 1998; Toy et al. 1998). Of these, *Six3* is specifically expressed in the developing eye and ventral forebrain. Recently, in *Drosophila* a member of the *Six* subclass of homeobox genes, *optix*, has been isolated, which by sequence comparison of the homeobox appears to be the ortholog of *Six3* (Toy et al. 1998). Functional analysis of *optix*, however, has not yet been reported.

In medaka fish (*Oryzias latipes*) *Six3* is expressed in the anterior-most neuroectoderm at gastrula stages and later in the developing retina (Loosli et al. 1998). Mosaic misexpression of mouse *Six3* in small clones, in response to injected plasmid DNA, resulted in the formation of ectopic lenses in the region of the otic vesicle, suggesting a decisive role for *Six3* during vertebrate lens development (Oliver et al. 1996). Considering the expression of *Six3* in the retinal primordia and the essential role of a *Drosophila* homolog, *so*, in *Drosophila* eye development, we investigated a potential role of *Six3* in early vertebrate retina development.

Results

We injected medaka *Six3* and mouse *Six3* RNA, respectively, into a single blastomere of medaka embryos at the two-cell stage. This led to a widespread and premature expression of *Six3* in the injected embryo. In response to both medaka *Six3* RNA (38 of 93) and mouse *Six3* RNA (19 of 47), injected embryos developed a dramatically enlarged optic vesicle at somitogenesis stages (stage 21; Iwamatsu 1994) suggesting a hyperplasia of retinal tissue (Fig. 1A–C). To unambiguously establish the identity of the ectopic tissue, we examined the expression of the retina-specific homeobox gene *Rx2* (Mathers et al. 1997). In wild-type embryos, *Rx2* is expressed exclusively in the presumptive neural retina and pigmented retinal epithelium (PRE) within the optic vesicle from the two-somite stage (stage 19) onward and subsequently becomes restricted to the developing neural retina in the optic cup (Fig. 1C). Thus, cells initially expressing *Rx2* contribute either to neural retina or to PRE and therefore *Rx2* serves as a specific marker for early retinal development. In the *Six3* induced enlarged optic vesicles we found an expansion of the *Rx2* expression domain, indicating that this tissue is of retinal identity (Fig. 1A,B). In addition, transverse sections of injected embryos revealed a twofold increase of the cell number in the enlarged optic vesicles (Fig. 3D,E, below), confirming retinal hyperplasia. The severity of retinal hyperplasia was always tightly correlated with the distribution of the coinjected lineage tracer, humanized green fluroescent protein (hGFP), in the injected embryos (Fig. 3F,G, below). These findings are consistent with the observation that overexpression of *Xenopus Six3* RNA leads to an enlarged retina in *Xenopus* embryos (M. Zuber, M. Perron, and W.A. Harris, pers. comm.). As additional morphological alterations we observed a broadening of the presumptive midbrain (medaka *Six3*, 43 of 93; mouse *Six3*, 15 of 47), which was in some cases associated with retinal hyperplasia.

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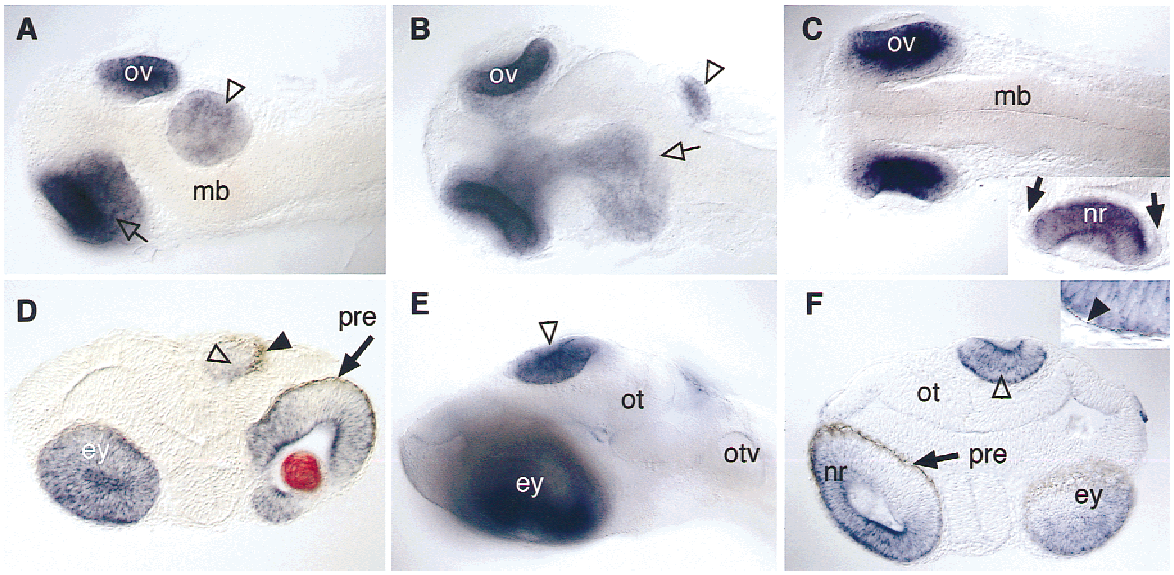

Figure 1. *Six3* overexpression results in the formation of ectopic retinal primordia and retinal hyperplasia. *Rx2* (blue) in situ analysis in control (*C*), medaka *Six3* (*A*,*D*) and mouse *Six3* (*B*,*E*,*F*) RNA-injected embryos, respectively; anterior is to the *left*. Open arrowheads indicate ectopic *Rx2* expression; open arrows indicate retinal hyperplasia. (*A–C*) Dorsal view of six-somite-stage embryos; (*D–F*) 32-somite-stage embryos. (*B*) Note the extent of retinal overgrowth (open arrow). (*C*) *Rx2* expression in the optic vesicle of a control embryo. Note that *Rx2* is not expressed in the dorsal diencephalon. (*Inset*) *Rx2* expression at 19-somite-stage confined to neural retina. Arrows point to prospective PRE. (*D*) Transverse section; open arrowhead indicates ectopic optic cup. Note ectopic PRE (arrowhead); arrow indicates wild-type PRE. Lens in wild-type eye is visualized by α-*A crystallin* expression (red). (*E*) Lateral view of embryo with optic cup-like structure (open arrowhead) in dorsal midbrain. (*F*) Transverse section at the level of the eye (open arrowhead in *E*); note ectopic optic cup (arrowhead); arrow indicates PRE; neural retina adjacent to PRE is free of *Rx2* expression. (*Inset*) Ectopic optic cup at higher magnification; arrowhead indicates ectopic PRE. (ey) Eye; (mb) midbrain; (nr) neural retina; (otv) otic vesicle; (ot) optic tectum; (ov) optic vesicle; (pre) pigmented retinal epithelium.

More importantly, we observed ectopic *Rx2* expression in *Six3*-injected medaka embryos concomitant with the onset of *Rx2* expression in the developing wild-type retina (stage 19; Fig. 1; data not shown). In all cases, ectopic *Rx2*-positive tissue was located exclusively in the midbrain and prospective cerebellum and was associated with a broadening of this region (medaka *Six3*, 20 of 93; mouse *Six3*, 10 of 47) (Fig. 1A,B). The widespread distribution of the injected RNA along the anteroposterior axis (Fig. 3F,G, below) indicates that retinal primordia can form in response to *Six3* expression at ectopic locations in a competent region of the brain.

At late organogenesis stages (34 somites, stage 29) ectopic *Rx2*-positive tissue was found to develop ectopic PRE in the dorsal midbrain of injected embryos, abutting ectopic *Rx2*-positive tissue (3 of 46; Fig. 1D–F). Transverse sections revealed an optic cup-like structure (Fig. 1, cf. inset in C with D and F). These ectopic optic cups consist of *Rx2*-expressing cells in a columnar arrangement (Fig. 1F inset), similar to the morphology of a neuroretina at this stage, surrounded by a single-cell layer of pigmented cells analogous to the PRE of the wild-type eye (Fig. 1D,F). In some cases, ectopic PRE was detected that was not associated with ectopic *Rx2* expression (3 of 46), similar to the wild-type situation in which the central region of the neural retina is free of *Rx2* expression (Fig. 1F). Thus, *Six3* overexpression leads to the formation of ectopic retinal primordia located in the region of the midbrain and prospective cerebellum, which have the potential to differentiate into both neural retina and PRE in ectopic optic cup-like structures.

In medaka *Six3* RNA-injected embryos, we observed in a few cases (2 of 98) an ectopic lens in the region of the otic vesicle (data not shown) but never in the vicinity of the ectopic retinas, consistent with our results of *Six3* plasmid DNA injections (Oliver et al. 1996). This suggests that the region competent to form ectopic retina in the brain (midbrain and prospective cerebellum) is not adjacent to the region competent to form an ectopic lens in the head ectoderm (level of rhombomere 5/6).

Among the Six family members the formation of retinal primordia is a specific feature of *Six3*, as another member of the vertebrate *Six* homeobox gene family, mouse *Six2*, had no effect on injection of the mRNA under conditions in which *Six3* initiates ectopic retina formation. In mouse *Six2* RNA-injected embryos, morphology, and expression of *Rx2* (0 of 114), *Pax6* (0 of 140) and *Six3* (0 of 113), respectively, were not affected. This is consistent with the finding that *Six2* is expressed in the neural retina and PRE of the adult mouse only (Oliver et al. 1995a,b; Kawakami et al. 1996a,b; Ohto et al. 1998), whereas *Six3* is expressed in the embryonic retinal primordia. Furthermore, in vitro binding studies indicate that *Six3* binds to a sequence unrelated to that of *Six2* (Kawakami et al. 1996b).

Overexpression of *Six3* in zebrafish embryos results in

ectopic *Emx2* expression, indicating an enlargement of the rostral forebrain (Kobayashi et al. 1998). To examine whether overexpression of *Six3* in medaka embryos similarly affects *Emx2* expression in injected embryos, we performed whole-mount in situ analysis. In the wild-type embryo *Emx2* is expressed dorsally in the anlage of the telencephalon and ventrally in the hypothalamus at the two-somite stage (Fig. 4C, below). *Six3* overexpression results in an enlarged *Emx2* expression domain (8 of 60), indicating an expansion of the forebrain in these embryos (Fig. 4D, below).

Pax6 expression in the developing retina has been shown to be required for retinal development (Hill et al. 1991; Hogan et al. 1986; Quinn et al. 1996). We therefore asked whether *Pax6* expression was induced upon *Six3* RNA injection. In wild-type embryos *Pax6* is expressed in an anterior domain, comprising the optic vesicles and diencephalon, and in a posterior domain that includes the posterior hindbrain and spinal cord at neurula stages; the midbrain and prospective cerebellum do not express *Pax6* (Fig. 2A; Loosli et al. 1998). Whole mount in situ hybridization of medaka *Six3* RNA-injected embryos revealed an expansion of the anterior *Pax6* expression domain and ectopic *Pax6* expression in the prospective midbrain and cerebellum at the late neurula stage (Fig. 2B,C; stage 18, 72 of 102), thus preceding ectopic *Rx2* expression in this region. At the six-somite stage (stage 21), ectopic *Pax6* expression covers the entire enlarged optic vesicles and extends into the midbrain and anterior hindbrain (medaka *Six3*, 58 of 79; mouse *Six3*, 24 of 29; data not shown). Isolated ectopic *Pax6*-expressing tissue was found within the midbrain and prospective cerebellum (Fig. 2D). This observation is consistent with the ectopic *Rx2* expression in this region. *Pax6* expression was not affected in the posterior hindbrain and spinal cord. Thus, *Six3* overexpression causes ectopic *Pax6* expression in a competent region corresponding to the midbrain and prospective cerebellum, preceding the formation of ectopic retinal primordia in this region.

To investigate whether *Pax6* is also expressed in the ectopic optic cups—as in the wild-type eye—we analyzed *Pax6* expression in *Six3*-injected embryos at the 34-somite stage. In 11 of 88 embryos ectopic PRE and closely associated *Pax6* expression was detected (Fig. 2E,F). The distribution of *Pax6* expression relative to the PRE in these structures was highly reminiscent of the wild-type retina at this stage (Fig. 2F and inset).

In *Drosophila*, it has been suggested that expression of a *Six3* homolog, *so*, is controlled by a regulatory feedback loop (Cheyette et al. 1994). In medaka embryos, injection of medaka or mouse *Six3* RNA equivalently resulted in the formation of ectopic retinal primordia and preceding marker gene expression (Figs. 1, cf. A with B, D with F, and 2, cf. B with C). To determine whether in vertebrates *Six3* functions in a regulatory feedback loop, we examined medaka *Six3* expression in embryos that were injected with mouse *Six3* RNA. In wild-type embryos, *Six3* is expressed in the forebrain and optic vesicles at neurula and somitogenesis stages (Fig. 3C; Loosli et al. 1998). At the late neurula stage in mouse *Six3* RNA-injected embryos, the endogenous *Six3* expression domain is expanded dramatically and endogenous *Six3* is expressed ectopically in the region of the midbrain and prospective cerebellum (31 of 40; data not shown). Also at the two-somite stage, ectopic medaka *Six3* expression was found in the midbrain and the prospective cerebellum (39 of 49) but not in the posterior hindbrain and spinal cord (Fig. 3A–C). Thus, ectopic medaka *Six3* expression is activated in the region where *Six3* misexpression results in ectopic *Pax6* expression (cf. Figs. 3B with 2, B and C).

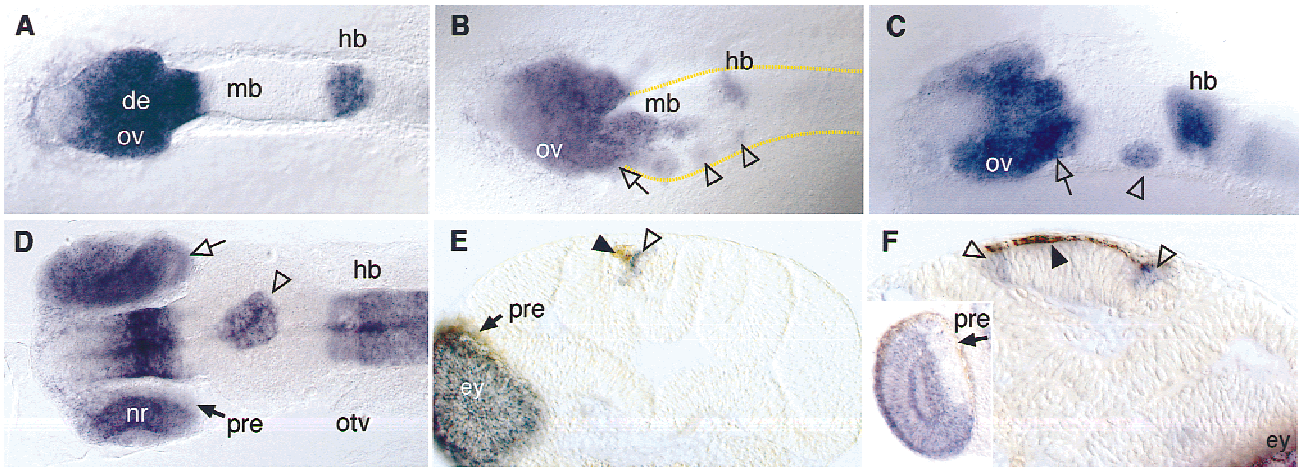

Figure 2. Ectopic *Pax6* expression in response to *Six3* overexpression. *Pax6* in situ analysis in control (*A*), medaka *Six3* (*C–F*), and mouse *Six3* (*B*) RNA-embryos. (*A,B*) Dorsal view of late neurula stage; (*C*) two-somite-stage embryos; (*D*) six-somite-stage embryos. (*E,F*) Transverse section of 34-somite stage embryos at the level of the midbrain. Open arrowheads indicate ectopic *Pax6* expression; open arrows retinal hyperplasia. (*B*) The embryonic axis is highlighted by yellow dotted lines. (*B,D*) Unilateral expansion of *Pax6* expression (open arrows) occurs in the optic vesicle. (*C,D*) Ectopic *Pax6* expression in the midbrain (open arrowhead). (*E,F*) Ectopic *Pax6* expression (open arrowhead) is abutting ectopic PRE (solid arrowhead) in the midbrain. (*F*) Only part of the ectopic retina expresses *Pax6*, similar to the wild-type situation (*inset*). (de) Diencephalon; (hb) hindbrain.

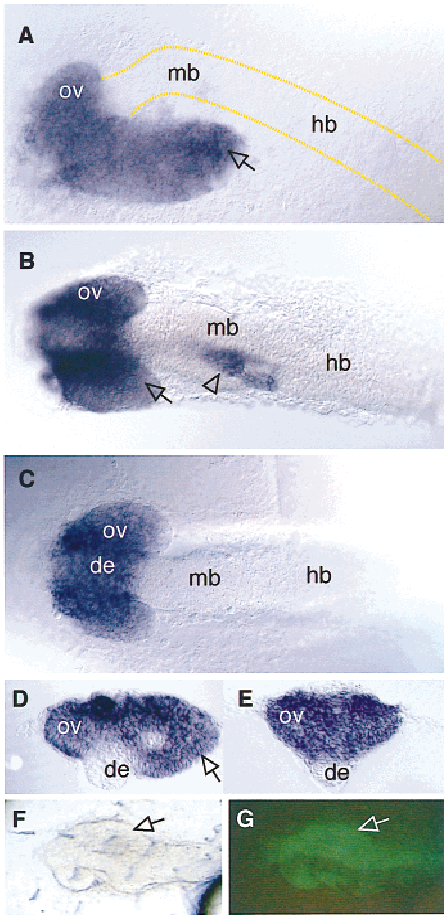

Figure 3. Mouse *Six3* expression reveals regulatory feedback control of the medaka *Six3* gene. *Six3* in situ (*A–E*) and lineage tracer (*F,G*) analysis in control (*C*), mouse (*A,B,D*), and medaka (*F,G*) *Six3* RNA-injected embryos. Dorsal views (*A–C,F,G*) and transverse sections at the level of the optic vesicle (*D,E*) of two-somite-stage embryos; (*A–C,F,G*) Anterior is to the *left*. Open arrowhead indicates ectopic *Six3* expression; open arrow indicates retinal hyperplasia. (*A*) The embryonic axis is highlighted by yellow dotted lines. (*F,G*) Lineage tracer analysis reveals a correlation of the severity of retinal hyperplasia with an unequal distribution of the injected RNA in the two body halves. Arrows point to enlarged optic vesicle, which is strongly fluorescent. (*G*) Widespread distribution of the lineage tracer along the entire anteroposterior axis.

Ectopic expression of the endogenous *Six3* gene in response to mouse *Six3* expression is indicative of a regulatory feedback loop involving the medaka *Six3* gene and suggests that this regulatory feature is conserved between vertebrates and invertebrates.

Ectopic *Pax6*, *Six3*, and the formation of retinal primordia in response to *Six3* overexpression was detected exclusively in the midbrain and prospective cerebellum. To investigate whether cells ectopically expressing *Pax6* and *Six3*, respectively, contribute to the ectopic retinal primordia as visualized by the expression of *Rx2*, we performed double labeling whole-mount in situ analysis. Similar to the wild-type situation in the developing retina, ectopic *Rx2* expression partially overlaps with both ectopic *Pax6* and *Six3* expression, respectively, at the six-somite stage (stage 21; Fig. 4). In all cases in which ectopic *Rx2* expression was detected, it was associated and partially overlapping with ectopic *Pax6* expression (11 of 47) and ectopic *Six3* expression (13 of 58).

Discussion

Our results provide evidence that *Six3* functions in vertebrate retina development. Overexpression of *Six3* causes an enlargement of the normal retina. Retinal hyperplasia is preceded by an expansion of the expression domains of *Six3* and *Pax6*, respectively. Consistent enlargement of structures derived from the retinal primordium in response to *Six3* overexpression was also observed in *Xenopus* (retinal hyperplasia; M. Zuber, M. Perron, and W.A. Harris, pers. comm.) and zebrafish (enlargement of the optic stalk region; Kobayashi et al. 1998). In addition to retinal hyperplasia we observed at a lower frequency an expansion of the forebrain in *Six3* RNA-injected embryos, which is in agreement with the findings in zebrafish (Kobayashi et al. 1998).

More importantly we show that early and widespread expression of *Six3* results in the formation of retinal primordia at ectopic locations in the midbrain and prospective cerebellum, involving a regulatory interaction of *Six3* and *Pax6*. Similar to the wild-type situation in the developing eye, *Pax6* and *Six3* are expressed in the region where the ectopic retinal primordia will subsequently form. These ectopic retinal primordia have the potential to develop into optic cups, as visualized by morphology and marker gene expression. The higher frequency of ectopic retinal primordia at early somitogenesis stages compared to ectopic optic cups formed at the 34-somite

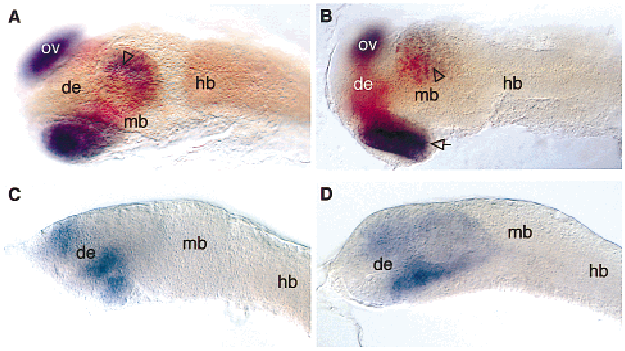

Figure 4. Ectopic *Rx2* expression partially overlaps with ectopic *Pax6* and *Six3* expression, respectively; expanded *Emx2* expression indicates enlarged forebrain. (*A,B*) Dorsal views of six-somite-stage embryos injected with medaka *Six3* RNA (*A*) and mouse *Six3* RNA (*B*), respectively. The focal plane is dorsal at the level of the optic tectum. (*A*) *Rx2* expression (blue) partially overlaps with *Pax6* expression (red) in the ectopic retinal primordium (open arrowhead) as in the wild-type eye. (*B*) *Rx2* expression (blue) partially overlapping with *Six3* expression (red) in the ectopic retinal primordium (open arrowhead) and the enlarged optic vesicle (open arrow), respectively. (*C,D*) Lateral views of control (*C*) and medaka Six3-injected embryos (*D*). Note expanded *Emx2* expression domain in *D*.

stage indicates that not all ectopic retinal primordia develop into an optic cup. Thus, *Six3* initiates, but not fully implements, later stages of retinal development.

Our results indicate that the region competent to form ectopic retinal primordia corresponds to the midbrain and prospective cerebellum, where *Pax6* is normally not expressed. In this region of competence ectopic *Six3* can activate *Pax6* expression. It is interesting to note that the region in which wild-type and ectopic retinal primordia develop lies within the expression domain of the *Otx* genes (Simeone et al. 1992; Li et al. 1994; Mori et al. 1994; Loosli et al. 1998), which based on their expression at early embryogenesis stages and function (Acampora et al. 1995; Matsuo et al. 1995; Ang et al. 1996) could play a role in providing competence for retinal development.

Ectopic lenses are formed in the region of the otic vesicle in response to *Six3* overexpression both by RNA as well as plasmid DNA injection. On the other hand, ectopic retinal primordia formation was observed exclusively in response to *Six3* RNA injection. This correlates well with the finding that overexpression of *Six3* by RNA but not DNA (Oliver et al. 1996) injection induces *Pax6* expression. Thus, early and widespread overexpression of *Six3* at high levels is sufficient for the induction of *Pax6* expression, whereas *Six3* expression in small clones starting at mid-blastula transition is not. The correlation of ectopic *Pax6* expression and retinal primordia formation suggests that *Pax6* expression is a prerequisite for ectopic retina formation, supported by the essential role of *Pax6* in eye development of vertebrates and invertebrates (Hill et al. 1991; Ton et al. 1991; Matsuo et al. 1993; Quiring et al. 1994; Halder et al. 1995a).

In *Drosophila* the *Six3* homolog *so* when coexpressed with *eyes absent*, can ectopically activate a *Pax6* homolog *eyeless* (*ey*), and cause ectopic eye formation in a *Pax6*-dependent manner (Pignoni et al. 1997). This hints at an evolutionary conserved interaction of homologous genes in vertebrates and invertebrates. In addition, our results demonstrate a regulatory feedback loop of *Six3* in developing retinal primordia, a mechanism that was suggested for *so* in *Drosophila*.

Thus, in addition to a structural conservation of the genes, their genetic interactions appear also to be conserved between vertebrates and invertebrates. It has been argued that in *Drosophila* an interactive network of eye specification genes, including *so* and *ey*, serves to implement and maintain the retinal cell fate program (Chen et al. 1997; Desplan 1997; Pignoni et al. 1997; Halder et al. 1998). Our experiments reveal that in vertebrates this developmental program involves evolutionary conserved interactions of *Six3* and *Pax6*, homologs of *so* and *ey*.

Materials and methods

Medaka stocks

Wild-type *O. latipes* from a closed stock at the MPI for Biophysical Chemistry were kept as described (Köster et al. 1997).

Isolation of partial Rx2 *and* Emx2 *cDNA*

A 560-bp fragment encoding medaka *Rx2* and a 540-bp fragment encoding medaka *Emx2* were amplified by RT–PCR from total RNA isolated from early neurula-stage (stage 17; Iwamatsu 1994) or early somitogenesis-stage (stage 19–21; Iwamatsu 1994) embryos, respectively, using degenerate PCR primers specific for *Rx2* (up, 5′-GACGACGAGCARCARCCIAARAARAARCA; low, 5′-AYRSHYTGDATRTGYTCYTTIGCYTTCAT) or *Emx* (up, 5′-ACIATCGAGWSIYTIGTIGGIAARGA; low, 5′-CYGTTYTGRAACCAAACYTTIACYTG). PCR conditions were 5 cycles at 94°C for 1 min, 48°C for 2 min, and 72°C for 4 min, followed by 30 cycles (35 cycles for *Emx2*) with annealing at 53°C. The resulting PCR products were cloned into the TopoTA vector (Invitrogen) and sequenced. The accession numbers in the EMBL database are AJ007939 and AJ132403.

Plasmids

A 1.3-kb *Apa*I–*Hin*dIII fragment of the medaka *Six3* cDNA (Loosli et al. 1998) and a 0.75-kb fragment of hGFP (Clontech), respectively, were cloned into the pCS2 vector. The entire cDNA of the mouse *Six3* cDNA (1.4 kb; Oliver et al. 1995a) and the mouse *Six2* cDNA (2.2 kb; Kawakami et al. 1996b), respectively, were cloned into the pCS2 vector. The resulting vectors were analyzed by digestions with restriction enzymes and the correct orientation was verified by sequence analysis. Expression of a protein of the expected size was confirmed by in vitro translation.

RNA injections

Linearized pCS*mouseSix3*, pCS*medakaSix3*, pCS*hGFP*, and pCS*mouseSix2* plasmid DNA was in vitro transcribed (Ambion SP6 mMessage mMachine Kit). The purified RNA (Qiagen RNeasy Kit) was injected in 1× Yamamoto ringer (Yamamoto 1975) into one blastomere at the two-cell stage as described (Köster et al. 1997). Injected embryos were raised as described at 28°C (Köster et al. 1997). An aliquot of the injection solution was analyzed by agarose gel electrophoresis to verify the concentration and integrity of the RNA. We have examined the effects of *Six3* overexpression over a wide concentration range (35–200 ng/µl both for medaka and mouse *Six3* RNA). We observed formation of ectopic retinal primordia and retinal hyperplasia over the entire concentration range. For the analysis presented, medaka *Six3* RNA and mouse *Six3* RNA, respectively, were injected at 100 ng/µl. Medaka *Six3* RNA was coinjected at 100 ng/µl with 30 ng/µl *hGFP* as a lineage tracer. The distribution of the GFP-protein was analyzed at the two somite stage by microscopy of live embryos. The morphology of the injected embryos was unaffected in 22 of 93 (medaka *Six3*) and 19 of 47 (mouse *Six3*), respectively. Mouse *Six2* RNA was coinjected at 200 and 500 ng/µl with 60 ng/µl *hGFP* RNA, respectively. The integrity of the injected RNAs was verified by fluorescence microscopy of live embryos at neurula stages.

For controls, *hGFP* RNA was injected at 200 and 400 ng/µl, respectively. To test the coding capacity of the injected RNA, fluorescence was examined at neurula stages by microscopy of live embryos. Fluorescent embryos (>95%) were fixed for whole-mount in situ hybridization.

Whole-mount in situ hybridization and vibratome sectioning

Whole-mount in situ hybridization was performed using digoxigenin- and fluorescein-labeled RNA riboprobes as described (Loosli et al. 1998). The entire cloned cDNAs of *Pax6*, *Six3*, *Rx2*, *Emx2*, and α-*A crystallin*, respectively were transcribed for the RNA riboprobes. Vibratome sections were done following standard procedures (Bober et al. 1994). Retinal hyperplasia was quantified by counting cell numbers in transverse sections at the comparable levels of mouse *Six3* RNA injected ($n = 3$; Θ 182 cells) and control embryos ($n = 4$; Θ 84 cells), respectively.

In vitro transcription/translation

Medaka *Six3*, mouse *Six3*, and mouse *Six2* RNA, respectively, were in vitro translated to test their coding capacity. In vitro transcription and translation was performed according to the manufacturer's instructions (Promega). One microgram of pCS*mouseSix3*, pCS*medakaSix3*, and pCS*mouseSix2* plasmid, respectively, SP6 RNA polymerase (Boehringer), rabbit reticulocyte lysate (Promega), and [^{35}S]methionine were used, products were separated by SDS–PAGE, and visualized by autoradiography. Proteins of the expected size were detected for medaka *Six3*, mouse *Six3*, and mouse *Six2*, respectively.

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Note added in proof

While this manuscript was in press, we were informed by W.A. Harris that the isolated *Xenopus* gene mentioned in the text is the *Optx2* homolog.

References

Acampora, D., S. Mazan, Y. Lallemand, V. Avantaggiato, M. Maury, A. Simeone, and P. Brulet. 1995. Forebrain and midbrain regions are deleted in Otx2–/– mutants due to a defective anterior neuroectoderm specification during gastrulation. *Development* **121:** 3279–3290.

Ang, S., O. Jin, M. Rhinn, N. Daigle, L. Stevenson, and J. Rossant. 1996. A targeted mouse Otx2 mutation leads to severe defects in gastrulation and formation of axial mesoderm and to deletion of rostral brain. *Development* **122:** 243–252.

Bober, E., T. Franz, H.H. Arnold, P. Gruss, and P. Tremblay. 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.

Bovolenta, P., I.A. Mallamac, L. Puelles, and E. Boncinelli. 1998. Expression pattern of cSix3, a member of the six/sine oculis family of transcription factors. *Mech. Dev.* **70:** 201–203.

Chen, R., M. Amoui, Z. Zhang, and G. Mardon. 1997. Dachshund and eyes absent proteins form a complex and function synergistically to induce ectopic eye development in Drosophila. *Cell* **91:** 893–903.

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

Desplan, C. 1997. Eye development: Governed by a dictator or a Junta? *Cell* **91:** 861–864.

Grainger, R.M. 1996. New perspectives on embryonic lens induction. *Semin. Cell Dev. Biol.* **7:** 149–155.

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

———. 1995b. New perspectives on eye evolution. *Curr. Opin. Genet. Dev.* **5:** 602–609.

Halder, G., P. Callaerts, S. Flister, U. Walldorf, U. Kloter, and W.J. Gehring. 1998. Eyeless initiates the expression of both sine oculis and eyes absent during Drosophila compound eye development. *Development* **125:** 2181–2191.

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

Hogan, B.L.M., G. Horsburgh, J. Cohen, C.M. Hetherington, G. Fisher, and M.F. Lyon. 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. *Zoo. Sci.* **11:** 825–839.

Kawakami, K., H. Ohto, K. Ikeda, and R.G. Roeder. 1996a. Structure, function and expression of a murine homeobox protein AREC3, a homolog of Drosophila sine oculis gene product, and implication in development. *Nucleic Acids Res.* **24:** 303–310.

Kawakami, K., H. Ohto, T. Takizawa, and T. Saito. 1996b. Identification and expression of six family genes in mouse retina. *FEBS Lett.* **393:** 259–263.

Kobayashi, M., R. Toyama, H. Takeda, I.B. Dawid, and K. Kawakami. 1998. Overexpression of the forebrain-specific homeobox gene six3 induces rostral forebrain enlargement in zebrafish. *Development* **125:** 2973–2982.

Köster, R., R. Stick, F. Loosli, and J. Wittbrodt. 1997. Medaka spalt acts as a target gene of hedgehog signaling. *Development* **124:** 3147–3156.

Li, Y., M.L. Allende, R. Finkelstein, and E.S. Weinberg. 1994. Expression of two zebrafish orthodenticle-related genes in the embryonic brain. *Mech. Dev.* **48:** 229–244.

Loosli, F., R.W. Köster, M. Carl, A. Krone, and J. Wittbrodt. 1998. Six3, a medaka homolog of the Drosophila homeobox gene sine oculis is expressed in the anterior embryonic shield and the developing eye. *Mech. Dev.* **74:** 159–164.

Mathers, P.H., A. Grinberg, K.A. Mahon, and M. Jamrich. 1997. The Rx homeobox gene is essential for vertebrate eye development. *Nature* **387:** 603–607.

Matsuo, T., N. Osumi-Yamashita, S. Noji, H. Ohuchi, E. Koyama, F. Myokai, N. Matsuo, S. Toniguchi, H. Dari, S. Jseki, Y. Ninomiya, M. Fujiwara, T. Watanabe, and K. Eto. 1993. A mutation at the *Pax-6* gene in rat small eye is associated with impaired migration of midbrain crest cells. *Nat. Genet.* **3:** 299–304.

Matsuo, I., S. Kuratani, C. Kimura, N. Takeda, and S. Aizawa. 1995. Mouse Otx2 functions in the formation and patterning of rostral head. *Genes & Dev.* **9:** 2646–2658.

Mori, H., Y. Miyazaki, T. Morita, H. Nitta, and M. Mishina. 1994. Different spatio-temporal expressions of three otx homeoprotein transcripts during zebrafish embryogenesis. *Brain Res. Mol. Brain Res.* **27:** 221–231.

Ohto, H., T. Takizawa, T. Saito, M. Kobayashi, K. Ikeda, and K. Kawakami. 1998. Tissue and developmental distribution of six family gene products. *Int. J. Dev. Biol.* **42:** 141–148.

Oliver, G., A. Mailhos, R. Wehr, N.G. Copeland, N.A. Jenkins, and P. Gruss. 1995a. 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.

Oliver, G., R. Wehr, N. Jenkins, N. Copeland, B. Cheyette, V. Hartenstein, S. Zipursky, and P. Gruss. 1995b. Homeobox genes and connective tissue patterning. *Development* **121:** 693–705.

Oliver, G., F. Loosli, R. Köster, J. Wittbrodt, and P. Gruss. 1996. Ectopic lens induction in fish in response to the murine homeobox gene Six3. *Mech. Dev.* **60:** 233–239.

Pignoni, F., B. Hu, K.H. Zavitz, J. Xiao, P.A. Garrity, and S.L. Zipursky. 1997. The eye-specification proteins So and Eya form a complex and regulate multiple steps in Drosophila eye development. *Cell* **91:** 881–891.

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

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

Seo, H.C., O. Drivenes, S. Ellingsen, and A. Fjose. 1998. Expression of two zebrafish homologs of the murine Six3 gene demarcates the initial eye primordia. *Mech. Dev.* **73:** 45–57.

Simeone, A., D. Acampora, M. Gulisano, A. Stornaiuolo, and E. Boncinelli. 1992. Nested expression domains of four homeobox genes in developing rostral brain [see comments]. *Nature* **358:** 687–690.

Ton, C.C., H. Hirvonen, H. Miwa, M.M. Weil, P. Monaghan, T. Jordan, V. van Heyningen, N.D. Hastie, H. Meijers-Heijboer, M. Drechsler et al. 1991. Positional cloning and characterization of a paired box- and homeobox-containing gene from the aniridia region. *Cell* **67:** 1059–1074.

Toy, J., J.-M. Yang, G. Leppert, and O.H. Sundin. 1998. The Optx2 homeobox gene is expressed in early precursors of the eye and activates retina-specific genes. *Proc. Natl. Acad. Sci.* **95:** 10643–10648.

Xu, P.X., I. Woo, H. Her, D.R. Beier, and R.L. Maas. 1997. Mouse eye homologs of the Drosophila eyes absent gene require Pax6 for expression in lens and nasal placode. *Development* **124:** 219–231.

Yamamoto, T. 1975. *Medaka (killifish), biology and strains.* Keigaku Publishing Company, Tokyo, Japan.

Six3 overexpression initiates the formation of ectopic retina

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