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Expanded retina territory by midbrain transformation upon overexpression of *Six6* (*Optx2*) in *Xenopus* embryos

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Abstract

During vertebrate eye development, the expression of the homeobox gene Six6 is restricted to the neural retina and is initiated later than *Rx* and *Pax6* in the presumptive retina field. We show here that overexpression of mouse *Six6* in *Xenopus* embryos can induce transformation of competent tissue of the anterior neural plate into retinal tissue. In *Six6* injected embryos, the molecular identity of the presumptive midbrain and rostral hindbrain regions was lost, as shown by the absence of *XEn-2* and *Xpax2* expression, being replaced by the ectopic expression of the retinal markers *Xpax6* and *Xrx*. When allowed to grow further, *Six6* injected embryos developed ectopic eye-like structures in the rostral brain and showed a transformation of the midbrain into retina. Similar results were obtained upon overexpression of *Six3* or *Xsix3*, revealing a possible redundance of *Six3* and *Six6* activities. Taken together, results obtained suggest that during normal retina development, the relatively late expressed Six6 gene becomes part of a network of retinal homeobox genes that are linked together by positive feedback loops. Furthermore, our results demonstrate that the primitive neural ectoderm of the future midbrain and rostral hindbrain is competent to form retinal tissue.

Keywords: Six6; *Optx2*; *Six3*; Homeobox; Retina; Eye development; Midbrain; *Xenopus*

1. Introduction

In avian embryos, the anterior neural plate contains precursor cells which give rise to most of the tissue components of the future rostral portion of the head. These include non-neural derivatives, such as the olfactory placodes, ectoderm of the nasal cavity and adenohypophysis, as well as neural derivatives, such as the hypothalamus, telencephalon, diencephalon, neurohypophysis, optic stalk and optic vesicles (Couly and Le Douarin, 1988). The origin of retinal stem cells within this area and the question as to how they become committed to the retinal program is not well understood. Expression pattern analysis of *Xpax6* and *ET* genes in *Xenopus* embryos, combined with graft experiments, suggests that a single morphogenetic field, referred to as the retina field, gives rise to two separate, laterally located retina forming groups of cells (Li et al., 1997). In this model, the retina field, which is localized in the anterior neural plate, is divided by suppression of retina formation in the median portion of the field by a diffusible morphogen emanating from the pre-chordal plate. One of the best candidates for this function is *Sonic hedgehog* (*Shh*). In humans and in the mouse, *SHH* mutations lead to severe midline defects and to cyclopean eyes, i.e. the presence of a single eye in the middle of the forehead (Chiang et al., 1996; Roessler et al., 1996). In addition, *Shh* injections into zebrafish embryos result in an enlargement of the *Pax2* expression domain in the optic stalk, while the *Pax6* expression domain decreases in the optic vesicle, suggesting a role for *Shh* in retina field resolution and optic stalk progression (Ekker et al., 1995; Macdonald et al., 1995).

In the mouse, only few genes are known to be expressed both in the anterior neural plate and then later in the optic vesicles, and are therefore potentially involved in retina stem cell formation or specification. *Otx2*, one of the two murine homologues of the *Drosophila* homeobox gene *orthodenticle*, is expressed in the anterior neuroectoderm of the embryo and, by 9.5 d.p.c., demarcates forebrain and midbrain regions with a sharp boundary at the midbrain/hindbrain junction (Simeone et al., 1992, 1993). *Otx2* is

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also expressed in the optic vesicle, and thus represents one of the earliest genes that demarcates the presumptive retina territory. *Otx2* homozygous mutant embryos show truncation of the forehead at the rostral hindbrain position and *Otx2* heterozygous mutant embryos exhibit severe forehead anomalies, including microphthalmia and anophthalmia (Acampora et al., 1995; Matsuo et al., 1995).

At the neural plate stage, the homeobox genes *Rx*, *Six3* and *Pax6* demarcate the retina field and are also later found to be expressed in the retina. *Rx* has been shown to be essential for optic sulcus and ventral forebrain formation in the mouse, and *Rx* RNA mis-expression in *Xenopus* embryos is sufficient to induce ectopic RPE (retinal pigmented epithelium) formation between the eyes and the anterior neural tube (Furukawa et al., 1997; Mathers et al., 1997). *Six3* is a member of the *sine oculis* (Cheyette et al., 1994) family of homeoproteins and it is one of the two orthologues of the *Drosophila optix* gene (Oliver et al., 1995; Toy et al., 1998). In mouse embryos, *Six3* is expressed in the most anterior portion of the neural plate and in the developing eyes (Oliver et al., 1995). Overexpression experiments in medaka and in zebrafish showed that *Six3* can induce the formation of ectopic lens tissue in the region of the otic placode, as well as an enlargement of the rostral forebrain (Oliver et al., 1996; Kobayashi et al., 1998). *Pax6* is a member of the paired domain containing gene family (Walther and Gruss, 1991) and it is conserved from fly to man (for review see Halder et al., 1995a; Oliver and Gruss, 1997). In *Drosophila*, *eyeless* is sufficient to induce ectopic eye formation on wings, legs and antennae and it is required for the development of the compound eye (Quiring et al., 1994; Halder et al., 1995b). In mouse and human, *PAX6* heterozygous mutations lead to the *small eye* (*Sey*) phenotype and to Aniridia, respectively, and *Pax6* homozygous mutations result in the complete absence of eye formation (Hogan et al., 1986; Hill et al., 1991; Ton et al., 1991). Finally, mice mutant for the Lim homeobox gene *Lhx2* have no eyes and present severe ventral forebrain defects (Porter et al., 1997).

We have recently reported on the isolation of a novel murine *Six3*-related gene, *Six6*. Mouse Six6 and Six3 share strong amino-acid conservation in their Six domain and homeobox, but are highly divergent in their amino- and carboxy-terminal regions. In addition, Six3 contains a long amino-terminal region that is not present in Six6 (Oliver et al., 1995; Jean et al., 1999). During mouse development, *Six6* is expressed sequentially in the pituitary/hypothalamic axis, the ventral optic stalk and in the neural retina, but not in the RPE and in the lens (Jean et al., 1999). In contrast to *Pax6*, *Rx* and *Six3*, *Six6* was not found to be expressed in the retina field at the neural plate stage, suggesting a rather 'late' function for *Six6* in retina formation. *Six6* was also isolated in the chicken and in *Xenopus* as *Optx2* (*XOptx2*) (Toy et al., 1998; Lopez-Rios et al., 1999; Zuber et al., 1999). In addition, a closely related partial cDNA clone has been isolated in *Drosophila* as *optix* (Toy et al., 1998). In the *Xenopus* embryo, *XOptx2* was reported to be expressed at stage 14–15 in the anterior neural ridge (presumptive pituitary/hypothalamic axis) and by stage 17 in the ventral diencephalon, the optic stalk and the ventral portion of the eye field (Zuber et al., 1999). *XOptx2* is thus expressed later than *Xpax6* and *Xrx* in the presumptive retina field (Li et al., 1997; Mathers et al., 1997) and shows an expression pattern that is merely identical to chicken and mouse *Six6* (*Optx2*) during development (Jean et al., 1999; Lopez-Rios et al., 1999). Finally, Six6 (Optx2) amino-acid sequence and protein structure are highly conserved in all the vertebrate species where it has been isolated (see Zuber et al., 1999).

Recent RNA injection experiments in the medaka showed that mouse or medaka *Six3* can induce ectopic expression of *Pax6*, *Rx* and *Six3* in the midbrain in a patch-like pattern, leading to formation of ectopic optic cup-like structures (Loosli et al., 1999). In contrast, RNA injection of *Xenopus Six6* (*Xsix6*/*XOptx2*) into *Xenopus* embryos suggests that *XOptx2* is involved in the control of retina proliferation and eye size regulation, but not in retina cell fate determination (Zuber et al., 1999). We report here that injection of low concentrations of either mouse *Six6*, mouse *Six3* or *Xenopus Six3* (*Xsix3*) into *Xenopus* embryos leads to an increase in retina size, and thus to an enlarged eye. We also demonstrate that injection of higher RNA concentrations of either of these genes results in the transformation of midbrain and rostral hindbrain into a tissue expressing retinal markers that has lost midbrain and midbrain/hindbrain identity markers. Finally, we show that *Six6* injection ultimately leads to the formation of ectopic eye-like structures and to the transformation of the midbrain into retina. Thus, the effects obtained upon ectopic expression of either *Six6* (*Optx2*) or *Six3* in *Xenopus* embryos are highly similar. The finding that the late expressed *Six6* can induce earlier genes involved in retina formation suggests that early and late activities are linked via a complex network that includes positive feedback loops. In addition, our finding demonstrates that the primitive neural ectoderm of the future midbrain and rostral hindbrain is competent to form retinal tissue.

2. Results

2.1. RNA injection of Six6, Six3 or Xsix3 at low concentrations induces an increase in retina size

We compared the phenotype of tailbud stage *Xenopus* embryos that were injected with low concentrations (10–25 pg) of *Six6*, *Six3* or *Xsix3* RNA into one blastomere at the two-cell stage. At these low RNA concentrations, no obvious effects were observed on the general morphology of the embryos, with the exception of a small increase in eye size (50–60% of the embryos), as visualized by in situ hybridization with retinal markers. As shown in Fig. 1, injections with either mouse *Six6*, mouse *Six3* or *Xenopus Six3* RNA

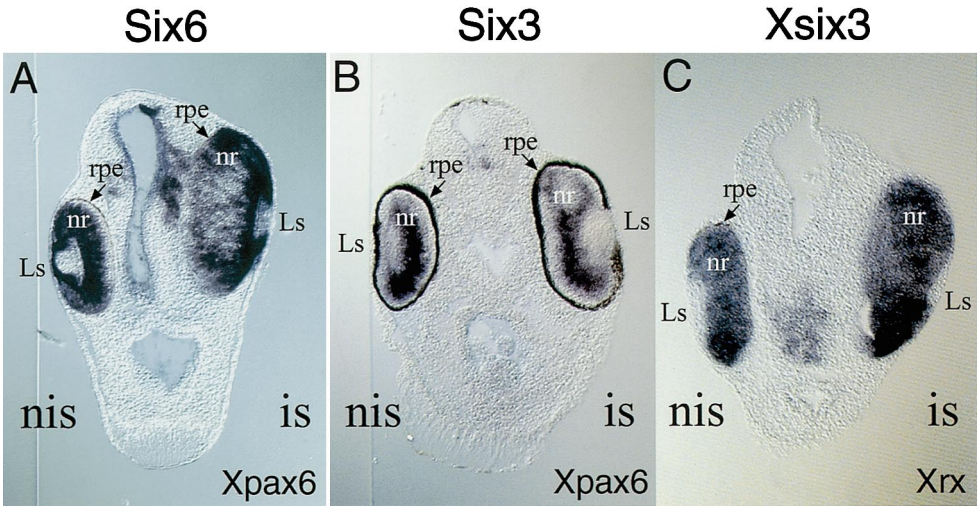

Fig. 1. RNA injection of *Six6*, *Six3* or *Xsix3* at low concentrations induces an increase in retina size. Vibratome sections of tailbud stage *Xenopus* embryos after whole-mount in situ hybridization. (A) Injection of 12.5 pg of *Six6* RNA, or (B) 10 pg of *Six3* RNA, or (C) 25 pg of *Xsix3* RNA leads to enlargement of the neural retina. Overall eye shape and lens formation are not affected. (A,B) *Xpax6* riboprobe. (C) *Xrx* riboprobe. rpe, retinal pigmented epithelium; nr, neural retina; ls, lens; is, injected side; nis, non-injected side.

induced an at least two-fold enlargement of the retina, when compared with the retina in the uninjected side. We also observed a reduction of the number of RPE cells in some of the sectioned embryos. Note that the overall morphology of the eye remains normal, including the presence of a lens. These results show that, at low concentrations, mouse *Six6* injection into *Xenopus* embryos induces the same effects as those reported for *Xenopus Six6* (*Xsix6*/*XOptx2*) (Zuber et al., 1999). They further reveal that, at these low RNA concentrations, injection of *Six3* or *Xsix3* mimics the effects obtained with *Six6*.

2.2. Six6, Six3 or Xsix3 at high concentrations leads to retinal expansion by midbrain transformation

The effects of high concentrations of *Six6* or *Six3* (75–200 pg of the corresponding mRNA) on the pattern defined by molecular markers for eye development were examined by whole-mount in situ hybridization at the neural groove stage (stage 18). In the developing *Xenopus* eye, *Xpax2* transcripts are detected in the region of the presumptive optic stalk and of the future midbrain/hindbrain boundary (Fig. 2A) (Heller and Brändli, 1997). In *Six6*, *Six3* or *Xsix3* injected embryos, the *Xpax2* expression domain extended into the presumptive midbrain territory and disappeared at the presumptive midbrain/hindbrain boundary (Fig. 2B–D). At this stage, *Xpax6* is normally expressed in the anterior neural plate including the eye field and also demarcates the forebrain/midbrain boundary; a second, more posterior expression domain is detected in the hindbrain and the future spinal cord (Fig. 2E) (Hollemann et al., 1998). *Six6*, *Six3* or *Xsix3* overexpression results in an expansion of the *Xpax6* expression domain in the eye and forebrain regions. It also induces ectopic expression of *Xpax6* in the presumptive midbrain area, leading to a fusion with its normal posterior expression domain (Fig. 2F–H). The frequency and intensity of *Xpax6* ectopic expression in the midbrain area were observed to be consistently higher upon Six6 injection when compared with *Six3* or *Xsix3* injection (compare Fig. 2F with Fig. 2G,H). We also observed a broader *Xpax6* expression domain in the neural folds upon *Six6*, *Six3* or *Xsix3* injection, which most likely reflects an increased proliferation of the neural tissue. The ectopic *Xpax6* and *Xpax2* expression domains in the presumptive midbrain area overlap at this stage, suggesting that this tissue represents an uncommitted retinal primordium. The *Xrx* expression domain demarcates the forebrain and eye fields (Fig. 2I) (Furukawa et al., 1997; Mathers et al., 1997). In *Six6*, *Six3* or *Xsix3* injected neural groove stage embryos, the *Xrx* expression domain is enlarged and found to expand into the presumptive midbrain area (Fig. 2J–L).

Xsix3, like its mouse homologue (Oliver et al., 1995), demarcates the presumptive optic field and forebrain area (Zhou et al., in preparation). In *Six6* ($n = 40$) injected embryos, the *Xsix3* expression domain was enlarged in the optic area, but the same gene was not found to be ectopically expressed in the presumptive midbrain area (Fig. 2R). Similar results were obtained upon *Six3* injections ($n = 25$). In addition, few embryos ($n = 3$) showed an expanded expression domain in the presumptive midbrain region (Fig. 2S). Altered midbrain/hindbrain patterning in the injected embryos was further reflected by the *Krox20* and *Engrailed2* (*Xen2*) expression patterns. *Xen2* demarcates the caudal midbrain and the midbrain/hindbrain boundary (Hemmati-Brivanlou et al., 1991), while *Xkrox20* is expressed in the hindbrain rhombomeres 3 and 5 in neural groove stage embryos (Bradley et al., 1993). Overexpression of *Six6*, *Six3* or *Xsix3* resulted in the loss of *Xen2* expression (Fig. 2N–P). However, segmentation of the hindbrain seems not to be affected, since the position of the

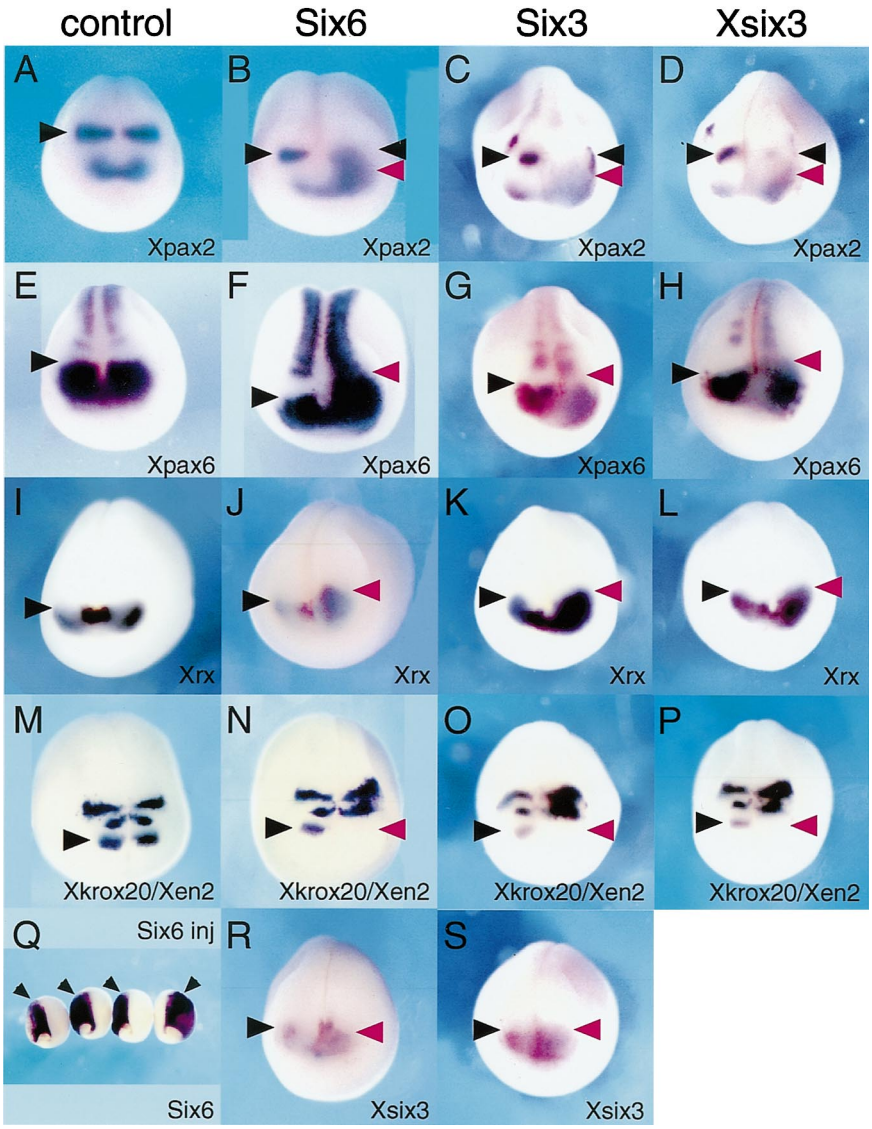

Fig. 2. RNA injection of *Six6*, *Six3* or *Xsix3* at high concentrations leads to retinal expansion by midbrain transformation. Whole-mount in situ hybridization of neural groove stage embryos (stage 18; anterior view). (A,E,I,M) Non-injected control embryos. (B,F,J,N,Q,R) *Six6* injected embryos. (C,G,K,O,S) *Six3* injected embryos. (D,H,L,P) *Xsix3* injected embryos. The injected side is always on the right (except for Q). (B–D) Ectopic expression of *Xpax2* in the optic area extends into the presumptive midbrain territory (red arrowhead). Expression at the midbrain/hindbrain boundary is lost (black arrowhead). (F–H) Enlarged *Xpax6* expression domain in the eye primordia and ectopic expression in the presumptive midbrain area (red arrowhead). The forebrain/midbrain boundary (black arrowhead) normally demarcated by *Xpax6* is lost. (J–L) Expanded *Xrx* expression in the optic area and ectopic expression in the presumptive midbrain territory (red arrowhead). Normal limit of *Xrx* posterior expression domain (black arrowhead). (N–P) *Xen2* expression at the midbrain/hindbrain boundary is absent (red arrowhead), while *Xkrox20* expression in the hindbrain rhombomeres 3 and 5 is maintained. Normal *Xen2* expression domain at the midbrain/hindbrain boundary (black arrowhead). (Q) *Six6* injected mRNA is still present in the injected side of the embryos at the neurula stage, as visualized with an antisense *Six6* riboprobe (arrowhead). (R) *Xsix3* expression domain (black arrowhead) remains limited to the optic area upon *Six6* injections (red arrowhead). (S) Moderate expansion of *Xsix3* expression domain into the midbrain area upon *Six3* injections (red arrowhead).

Xkrox20 expressing rhombomeres 3 and 5 is maintained (Fig. 2N–P). In most cases, an increase in the number of *Xkrox20* expressing cells can be detected on the injected side, which suggests increased proliferation (Fig. 2N–P). Thus, in addition to these proliferative effects, *Six6*, *Six3* and *Xsix3* overexpression leads to ectopic expression of *Xpax2*, *Xpax6* and *Xrx* in the presumptive midbrain and to the loss of midbrain and midbrain/hindbrain boundary identity in neurula stage embryos.

2.3. High concentrations of Six6 disrupt eye morphology, inhibit RPE formation and lead to anterior CNS hyperplasia

Inspection of *Xenopus* tadpoles injected with *Six6* at high concentrations revealed two different morphological defects: firstly, absence or reduction of RPE cells and body pigments on the injected side of the embryo (Figs. 3B,C and 4) and secondly, enlargement of the head and loss of eye formation (i.e. absence of lens and optic cup).

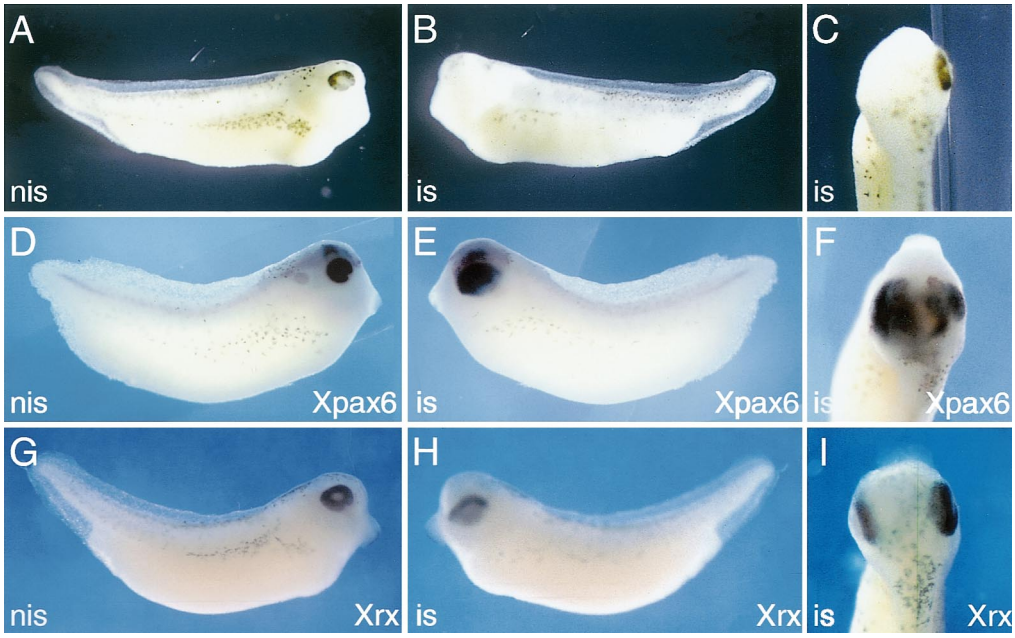

Fig. 3. *Six6* injection results in the repression of RPE formation and in the expansion of the expression domain of retinal markers. Left and right views of the same tadpole embryo (stage 36–38) are shown side by side. (C,F,I) Dorsal view of the head; anterior is at the top. (A–C) Loss of RPE and reduction of body pigmentation in the injected side (B). (D–F) Ectopic *Xpax6* expression expanding caudally and disruption of the normal eye and forebrain expression domains. (G–I) Enlarged *Xrx* expression domain in the optic area. is, injected side; nis, non-injected side.

The enlarged head phenotype was later found to be associated with an expanded *Xpax6* or *Xrx* expression domain (see below). Up to 100 pg *Six6* RNA results in a loss or reduction of eye pigmentation, while maintaining a relatively normal morphology; the use of higher *Six6* concentrations (200 pg) produced a higher proportion of embryos with axial defects and spina bifida phenotypes (Table 1).

Xpax6 is normally expressed in the eye (lens, retina and RPE), the forebrain, caudal hindbrain and spinal cord in tadpole (stage 36–38) *Xenopus* embryos (Fig. 3D,F). Overexpression of *Six6* results in a lateral and caudal expansion of the *Xpax6* expression domain accompanied by neural hyperproliferation in tadpole stage embryos (Figs. 3E,F and 4D–F). The ectopic *Xpax6* domain extends caudally to the position of the presumptive midbrain/hindbrain boundary and becomes located adjacent to the hindbrain (Fig. 4E,F). To further characterize the molecular nature of the tissue that ectopically expresses *Xpax6*, expression of *Xrx* was analyzed. Normally, *Xrx* expression is restricted to the retinal ciliary margin at tadpole stages of development (Mathers et al., 1997). In injected embryos, the *Xrx* expression domain is broadened within the normal eye territory (Fig. 3H,I) and extends into the presumptive midbrain and rostral hindbrain regions in a manner similar to *Xpax6* (Fig. 4G–I). *Xpax2* expression in the eye is restricted to the optic stalk in tadpole stage embryos. In contrast to the ectopic expression of *Xpax2* in the presumptive midbrain area at the neurula stage (Fig. 2B–D), no ectopic *Xpax2* expression could be detected at the tadpole stage in *Six6* injected embryos (Fig. 4A–C). This suggests that the initial retinal primordium tissue was now committed to a more advanced retinal fate. In addition, no *Xpax2* positive cells were found at the midbrain/hindbrain boundary on the injected side of the embryo, which is in contrast to the normal *Xpax2* expression in that region (Fig. 4C). Thus, overexpression of *Six6* resulted in enlarged *Xpax6* and *Xrx* expression

Table 1
Phenotypic effects of Six6 overexpression in *Xenopus* embryos

Six6 RNA injected (pg)	No. of experiments	No. of embryos (tadpole stage)	Reduction of retinal pigment (%)	Enlarged heads (%)[a]	Axial defects and spina bifida (%)
12	1	37	30	8	3
25	3	196	47	11	19
50	4	251	60	20	41
100	1	37	68	27	41
200	1	52	12	4	>80

[a] The head enlargement phenotype was associated with the reduction of retinal pigment.

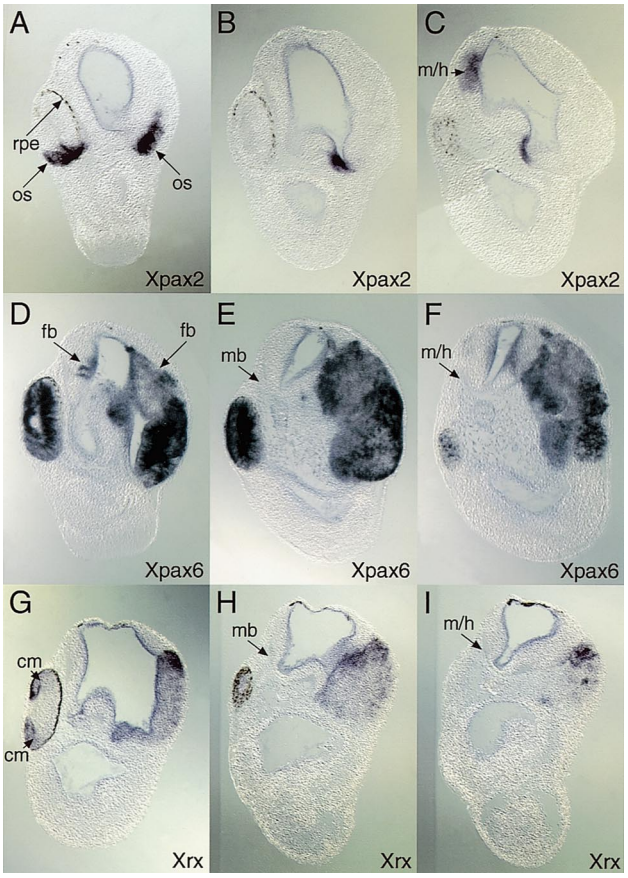

Fig. 4. Retinal hyperplasia and ectopic expression of *Xpax6* and *Xrx* as a result of *Six6* overexpression. Serial sections of tadpole embryos (stage 36–38) after whole-mount RNA in situ analysis are shown in a rostral to caudal direction within each row; the injected side is to the right. Note the strong reduction or absence of RPE on the injected side (A–C) in Six6 injected embryos (100 pg). *Xpax2* expression in the optic stalk is normal in the injected side (A,B), but expression is absent at the presumptive midbrain/hindbrain boundary (C). *Xpax6* expression is broader in the optic area and expands into the presumptive midbrain and rostral hindbrain areas (D–F). *Xrx* expression is normally restricted to the ciliary margin of the retina. At the injected side of the embryo, *Xrx* expression extends up to the presumptive rostral hindbrain region (G–I). fb, forebrain; mb, midbrain; m/h, midbrain/hindbrain boundary; os, optic stalk; rpe, retinal pigment epithelium; cm, ciliary margin.

domains that extend into the presumptive midbrain and rostral hindbrain areas at the tadpole stage of development. Consequently, the midbrain/hindbrain (isthmus) identity was lost. In addition, these injections induced a general brain overgrowth and disrupted normal eye and RPE formation.

In order to test whether these effects were specific to *Six6* activity, we also analyzed *Six3* and *Xsix3* injected embryos (50–800 pg of RNA) at the tadpole stage. In comparison to *Six6*, injection of 200 pg of either *Six3* or *Xsix3* RNA resulted in a much lower proportion of embryos with axial defects. However, a similar proportion of embryos (approximately 50%) presented a reduction or absence of RPE formation associated with hyperplasia of the optic and midbrain regions. These embryos also presented ectopic expression of *Xrx* and *Xpax6* in the midbrain area and an absence of *Xpax2* expression at the midbrain/hindbrain boundary (data not shown).

2.4. Six6 overexpression induces formation of ectopic eye-like structures and transformation of the midbrain into a retina

We performed further experiments to analyze if the *Six6*-transformed tissue with retinal characteristics retains the capacity to differentiate into RPE, or if it is terminally committed to the neural retina program. To this end, we allowed further growth up to stage 50 of development with Six6 injected embryos (100–200 pg) that were preselected for the main phenotype at the tailbud stage (reduction or absence of eye pigments and brain overgrowth). In more than 90% of the embryos, the normal eye was lacking on the injected side. When present, it was atrophic or abnormal. In one embryo (total $n = 58$), we observed the presence of two ectopic optic cup-shape structures containing a lens (Fig. 5A). More frequently, *Six6* injected embryos presented a single ($n = 20$) or two ($n = 3$) pigmented balls of cell closely associated with the rostral neural tube (Fig. 5B). Upon sectioning of five of these embryos, the pigmented structures were revealed to have a retinal morphology, with a layered neural retina surrounded by a RPE. Analysis of serial sections showed that the ectopic retinal tissue was fused to the forebrain (Fig. 5D; rostral section) and had replaced the midbrain (Fig. 5F,G; caudal section). In three out of the five sectioned embryos, a single lens was found always at the same rostral position and was sometime loosely associated with the ectopic retinal tissue (Fig. 5C).

These results show that *Six6* injection can ultimately lead to the formation of 'eye-like' structures in the rostral neural tube as well as to the transformation of the midbrain into a retina. Overall, these effects are reminiscent of what has been described for ectopic expression of *Six3* in medaka and of *Rx* and *Pax6* in *Xenopus* (Mathers et al., 1997; Chow et al., 1999; Loosli et al., 1999). Our results demonstrate that *Six6*-transformed tissue with retinal characteristics is not terminally committed to the neural retina program but can differentiate into RPE. Whether ectopic RPE formation by *Six6* overexpression occurred in a cell autonomous manner is not known. However, since *Six6* injection induces ectopic expression of *Rx* and *Pax6* at the neurula stage, it is likely that the final effects observed at stage 50 of development are not due to *Six6* per se, but are rather a consequence of the earlier activation of the genetic network controlling eye formation.

3. Discussion

We demonstrate that overexpression of either *Six6* or *Six3* in *Xenopus* embryos induces an increase in retina size at low concentrations. We also show that at higher concentrations, *Six6* or *Six3* overexpression induces the formation of an

enlarged *Xpax6* and *Xrx* positive tissue which extends up to the level of the rostral hindbrain. Use of molecular markers demonstrates that midbrain and rostral hindbrain cells have lost their normal identity and are reprogrammed into retinal cells. In support of this, we show that *Six6* can induce the formation of ectopic retinal structures in the mature midbrain. Finally, we observed that overexpression of *Six6* or *Six3* suppresses RPE formation in early embryos, suggesting either uncoupling between retina proliferation and retina differentiation or specific repressive activity of *Six6* and *Six3* on RPE formation. These results imply that *Six6* and *Six3* exert highly similar effects on retina proliferation and retina cell fate determination. On the basis of these observations, we propose that *Six6*, a gene expressed relatively late in the developing retina, is linked via a positive regulatory feedback loop to earlier expressed retinal genes in the process that leads to retina cell fate determination.

3.1. Six6 can induce cell fate conversion toward the retinal program

The most striking result that we have obtained upon overexpression of *Six6* is the 'conversion' of the presumptive midbrain and rostral hindbrain areas into tissue with retinal characteristics. The absence of a 'conversion' of other tissues by *Six6* overexpression implies that only specific territories of the early embryo are competent to carry out the execution of the retina program. This is reminiscent of the results obtained upon *Six3* injections in the medaka leading to ectopic lens tissue formation in the region of the otic vesicles (Oliver et al., 1996). In this case, only the otic placodes were found to be competent to be biased toward the lens program. In the case of *Six6* or *Six3* injections, the 'territory of retinal competence' ranged from the diencephalon to the rostral hindbrain and appears to exclude the most rostral portion of the forebrain, the caudal hindbrain and the spinal cord. This territory of the embryo is mainly defined by the expression domain of the homeobox genes *Otx1* and *Otx2* (Simeone et al., 1992, 1993). However, of these two genes, only *Otx2* is expressed early enough to allow for such a plasticity of the neuroectodermal cells, and targeted mutations do indeed suggest a direct role for *Otx2* in rostral head and eye patterning (see Section 1). Altogether these results suggest that *Otx2* may be involved in the establishment of retinal competence in the primitive neuroectoderm.

In addition to *Otx2*, several other genes are involved in retina formation. Specifically, targeted mutations in the mouse have demonstrated that *Rx*, *Pax6* and *Lhx2* are required for retina formation (see Section 1). These genes are normally expressed in the early forebrain but not in the midbrain. RNA in situ hybridization experiments showed that the midbrain has been replaced by *Xpax6* and *Xrx* positive cells in *Six6* injected embryos. These results demonstrate that *Six6*, like *Six3* (Loosli et al., 1999), can directly or indirectly activate *Xpax6* and *Xrx* expression in competent tissues. This observation is surprising since *Six6* is expressed later than *Pax6* and *Rx* during vertebrate retina development. Thus, *Six6* is likely to be involved in a regulatory feedback loop with these genes in order to execute the final retinal program (Fig. 6). Recent overexpression experiments in the medaka embryos have demonstrated that *Six3* can induce the formation of ectopic retina and ectopic optic cup surrounded by RPE in the midbrain and cerebellum (Loosli et al., 1999). These *Six3* injections did not affect normal eye formation in the optic area, although they induced obvious retina proliferation. In contrast, *Six6* or *Six3* overexpression in *Xenopus* embryos induces the formation of a relatively homogeneous retinal tissue from the optic area to the rostral hindbrain and disrupts normal eye and RPE formation. Furthermore, late *Six6* injected embryos ultimately developed an ectopic eye-like structure in the forebrain and midbrain, in a manner similar to *Six3* injected medaka embryos (Loosli et al., 1999). We also tested whether *Six6* could induce ectopic expression of *Xsix3*, as reported for mouse *Six3* in the medaka (Loosli et al., 1999). We found that although the *Xsix3* expression domain was enlarged in the optic area of *Six6* injected embryos, *Xsix3* expression was not ectopically induced. Similarly, mouse *Six3* injections induced ectopic expression of *Xsix3* very inefficiently when compared with induction of *Xrx*. These observations indicate that at least some of the effects observed differ depending on the model organisms used. We also demonstrate that *Six6*, *Six3* or *Xsix3* effects are dose-dependent: low concentration injections induce retina enlargement and high concentrations induce cell fate conversion. These results show that *Six6* (*Optx2*) does not only regulate retina or eye size by controlling cell proliferation (Zuber et al., 1999), but it is also involved in retina cell fate determination. From our results, it is also clear that the function of regulating eye size by means of controlling retina proliferation cannot be attributed to the activity of a single gene.

Previously, we have shown that *Six6* gene expression is maintained in the optic vesicle of *Pax6* homozygous mutant embryos (Jean et al., 1999). In addition, the expression of the *Rx*, *Six3* and *Lhx2* is also maintained in the optic vesicle of the *Pax6* mutants (G.B. and P.G., unpublished data). Finally, *Pax6* expression was reported to be present in the optic vesicles of *Lhx2* mutant embryos (Porter et al., 1997). These observations, together with the data presented here, suggest that retinal cell development in vertebrates requires a combinatorial code of homeobox genes. Such genes are interconnected by regulatory feedback loops but are not directly dependent on each other for their primary transcriptional activation. One possible explanation for such a system may be linked to the spatio-temporal expression pattern of each of these genes during normal development of the eyes (see Fig. 6). When their expression overlaps in a given portion of the embryo (i.e. the retina), activation of this regulatory network would force the cell to adopt final retinal identity. A comparable model has been proposed for

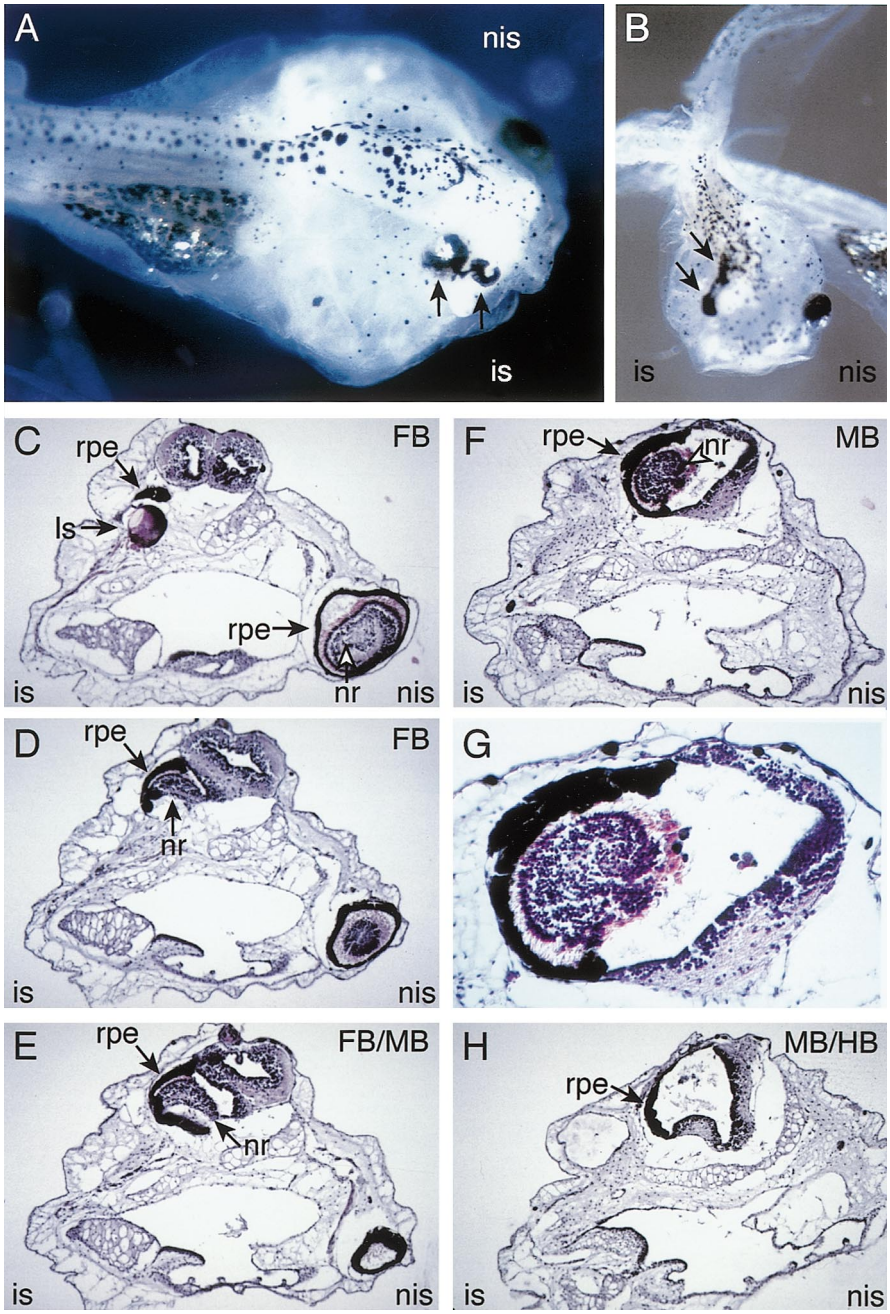

Fig. 5. *Six6* overexpression induces formation of ectopic eye-like structures and transformation of the midbrain into a retina. *Six6* injected embryos (100–200 pg) at stage 50 of development. (A) Presence of two optic cup-like structures that are adjacent to the rostral neural tube (arrows) and absence of normal eye formation at the injected side. (B) Formation of pigmented structures in the rostral neural tube at the injected side (arrows). (C–H) Cross serial sections of the same embryo from rostral to caudal (10 μm, H&E stained). The sections are spaced by an average of 60–80 μm. (C) Single ectopic lens in the rostral portion of the embryo. (D) Ectopic retinal tissue surrounded by a RPE that is fused to the forebrain. (F) Presence of a layered retinal tissue surrounded by a RPE that has replaced the normal midbrain tissue. (G) Higher magnification of (F). (H) No ectopic retina is found after the midbrain/hindbrain junction, except for some pigmented cells. rpe, retinal pigmented epithelium; nr, neural retina; ls, lens; is, injected side; nis, non-injected side; FB, forebrain; FB/MB, forebrain/midbrain; MB, midbrain; MB/HB, midbrain/hindbrain. Original magnification (C–F,H) 50×, (G) 100×.

the development of the compound eye in *Drosophila*. This was based on the observation that coexpression of *eye absent* with *sine oculis* or with *dachshund* leads to *eyeless* expression and to the formation of ectopic eyes (Chen et al., 1997; Pignoni et al., 1997), although *eyeless* is known to act 'upstream' of these genes. These results clearly demonstrate that vertebrates and insects share similar molecular mechanisms for eye formation.

3.2. Transdetermination versus proliferation?

RPE formation and pigmentation in general by *Six6* or

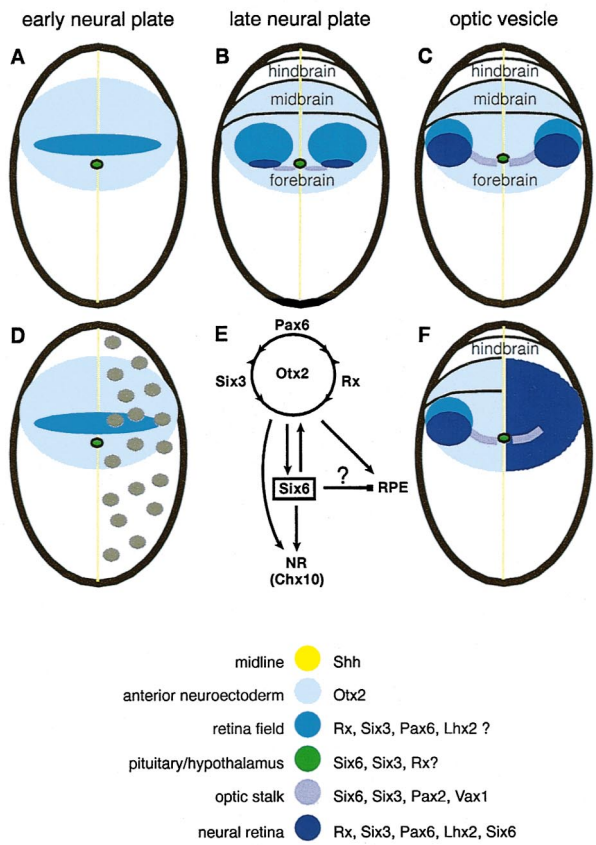

Fig. 6. A model for neural retina determination in vertebrates. (A–C) Sequential activation of genes involved in eye development from the early neural plate to the optic vesicle stages as observed normally. The diagram represents an anterior view of a schematized vertebrate embryo; the dorsal part is at the top. (A) *Rx*, *Pax6* and *Six3* demarcate the retina field and *Otx2* the anterior neuroectoderm of the future rostral head. (B) The retina field is divided by the activity of a morphogen (Shh) emanating from the midline region, concomitant with the appearance of the optic stalk. (C) *Six6* is expressed in the presumptive neural retina. (D–F) *Six6* directs competent tissue towards the retinal program. (D,E) Overexpression of *Six6* activates a regulatory feedback loop leading to ectopic expression of *Pax6* and *Rx* in the *Otx2* territory. (F) *Six6* overexpression leads to retinal expansion by midbrain transformation.

Six3 RNA injections is reduced or suppressed in a dose-dependant manner. This result correlates well with the absence of *Six6* expression in the mouse and chicken RPE and likewise with the capacity of *Six6* to induce the expression of neural retina markers such as *Chx10* when injected in RPE cultured cells (Toy et al., 1998; Jean et al., 1999; Lopez-Rios et al., 1999). How can this observation be reconciled with a regulatory feedback loop model involving, for example, *Pax6*, a gene expressed in the neural retina and in the RPE (Walther and Gruss, 1991)? One possibility may be that *Six6* is involved in the repression of downstream genes essential for neuroepithelium differentiation into RPE. However, the fact that *Six3* injections also repress RPE formation in *Xenopus* suggests that this effect might be unspecific. Another interpretation is that hyperproliferation of the retinal primordium is uncoupled from RPE differentiation. This is supported by the frequent absence of lens and optic cup formation in these injected embryos, suggesting that overall eye induction was abolished due to retina overproliferation. In addition, ectopic formation of RPE was observed in the injected embryos analyzed at stage 50, at a time where the overproliferative effects from the injection are no longer expected. Thus, it is not clear whether negative effects of *Six6* and *Six3* on RPE formation are due to retina overproliferation, specific RPE gene repression and/or transdetermination. We also show that *Six6* injections can lead to the formation of pigmented eye-like structures in the rostral brain when more mature embryos are examined. This suggests that *Six6*-transformed retinal tissue retains the ability to exit from the neural retina program when exposed to specific environmental cues. Finally, these observations indicate that *Six3* and *Six6* (*Optx2*) might have redundant functions in vivo.

In conclusion, we propose a model suggesting that *Pax6*, *Rx* and *Six3* act at the top of the genetic hierarchy governing retina formation in vertebrates and that *Otx2* is necessary for the establishment of retinal competence in the primitive neuroectoderm. The main function of *Six6* would be to execute the final retinal program and, by activating a regulatory feedback loop with these genes in the neural retina, establish the retinal identity of these cells. In that respect, *Six6* could act as a determinant of neural retina cell fate.

4. Materials and methods

4.1. Microinjection procedures

The full-length coding region of mouse *Six3* (Oliver et al., 1995) and *Xenopus Six3* (Zhou et al., in preparation) were cloned directly into the pCS2+ expression vector (Rupp et al., 1994). The full-length mouse *Six6* cDNA (Jean et al., 1999) was cloned into pCS2+ using a DNA fragment generated by PCR using primers harbouring the appropriate restrictions sites (F: 5′-GCG*GAATTC*ACGATGTTCCAGCTGCC-3′; R: 5′-CCG*CTCGAG*AGCTCAGATGTCGCACT-3′) and inserted into the *Eco*RI-*Xho*I site of the vector. The plasmid was checked by sequence analysis. Six6, Six3 and Xsix3 expression constructs were verified by in vitro translation assay. Capped RNA was transcribed using SP6 RNA polymerase as described (Kintner and Melton, 1987). RNA was injected in a volume of 5 nl at a concentration of 10–800 pg/nl into a single blastomere in embryos at the two-cell stage, as previously described (Coffman et al., 1990). Embryos were collected at the indicated stages and subjected to RNA in situ hybridization.

4.2. Whole-mount RNA in situ hybridization analysis and sectioning

In principle, whole-mount in situ hybridization was performed as described by Harland (1991) using digoxygenin-11-UTP-labelled antisense RNA probes. Preparation

of probes for *Xpax2* (Heller and Brändli, 1997), *Xpax6* (Hollemann et al., 1998), *Xrx* (Furukawa et al., 1997; Mathers et al., 1997), *Xkrox20* (Bradley et al., 1993) and *Xen2* (Hemmati-Brivanlou et al., 1991) was conducted as previously described. Digoxygenin-labelled hybrids were visualized by alkaline phosphatase-conjugated anti-digoxygenin Fab fragments and NBT/BCIP. NBT/BCIP stained embryos were afterward washed in methanol for 2 min prior to fixation. For sectioning, stained and post-fixed embryos were gelatine-embedded and sectioned at 30 μm thickness with a Vibratome 1000 (Technical Products International Inc.). The imaging of the mounted sections was performed on a Zeiss Axiophot with Normarski optics using a CCD camera (Sony).

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