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Retinoic acid-induced duplication of the zebrafish retina

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ABSTRACT **Exogenous treatment of zebrafish embryos with retinoic acid induces a duplication of the retinas during development. These effects occur only when retinoic acid is applied within a 2-hr period prior to and during the initial formation of the optic primordia, and they are concentration-dependent. Light microscopic examination reveals that the second retina derives from cells in the ventral region of the developing eyecup that normally become pigment epithelial cells. Two distinct ganglion cell fields are usually observed in eyes with duplicated retinas. Bundles of axons from each ganglion cell field join as they leave the eye and innervate the contralateral tectum.**

Retinoic (vitamin A) acid can cause striking alterations in limbs and other organs during embryonic development or regeneration. Beads impregnated with retinoic acid, when implanted within anterior positions of the developing limb bud, induce mirror-image digit duplications in the chicken limb (1, 2). Mirror-image duplication of digits is observed in regenerating limbs of larval amphibians immersed in retinoic acid-containing water (3–5). Retinoic acid can also induce duplication of the heart in the developing chicken (6). These observations are intriguing in light of experiments that have shown retinoic acid alters specific homeobox gene expression in *Xenopus*, chicken, and mouse embryos and induces the expression of several homeobox-containing *Hox* genes in embryonic carcinoma cells (for review, see refs. 7 and 8). The data suggest, therefore, that retinoic acid, or a close relative, is involved in specifying pattern formation during development, perhaps by the activation of certain *Hox* genes.

Although various substances, including retinoic acid, have been shown to have effects on the development of the brain (9–11), no agent has been found that induces duplication of specific central nervous system structures. We report here that retinoic acid, when applied exogenously to zebrafish embryos at particular times during development, induces a duplication of the retinas.

MATERIALS AND METHODS

Fish Maintenance and Embryo Collection. Breeding fish were maintained at 28°C on a 14-hr light/10-hr dark cycle. Prior to egg collection, breeding fish were placed in mesh nets (≈20 cm × 20 cm × 25 cm) that served as nuptial chambers. Fertilized eggs that fell through the nets were collected by siphoning from the bottom of the tank. Zero-hour fertilization was estimated to occur at the time natural spawning originated; that is, at the onset of light. Generally, eggs are laid within 30 min of light onset, but egg laying may be delayed by as much as 2 hr. To determine accurately the extent of development, individual embryos must be staged, using morphological criteria (12). Embryos were raised in tank water [made up in deionized water to which was added Instant Ocean (Aquarium Systems, Mentor, OH) at 100 mg/liter and Wardley's Bullseye pH regulator (Wardley Corp., Secaucus, NJ) at 1.3 ml/liter] in an incubator at 28°C.

Retinoic Acid Treatment. A stock solution of 0.1 M *all-trans*-retinoic acid (Sigma) was made up in dimethyl sulfoxide and kept in frozen aliquots at −20°C. The retinoic acid was thawed immediately before use and diluted to the appropriate concentration in tank water. Embryos were treated based on the time after fertilization and/or by carefully staged morphological criteria. After treatment, embryos were washed three times in tank water. Some embryos were raised in 0.2 mM phenylthiocarbamide (Sigma) in tank water to inhibit pigment formation. Control embryos received mock treatments with equivalent concentrations of dimethyl sulfoxide.

Light Microscopy. Embryos were fixed in 0.06 M sodium phosphate (pH 7.4) containing 1% paraformaldehyde, 2.5% (vol/vol) glutaraldehyde, 3% (wt/vol) sucrose, and 0.15 mM $CaCl_2$ for 2 hr at 4°C. The tissue was postfixed for 1 hr at 4°C in phosphate buffer containing 3% sucrose and 0.15 mM $CaCl_2$ with 1% OsO_4 added. After buffer wash, the tissue was dehydrated and embedded in an Epon–Araldite mixture. Thick sections (1–3 μm) were cut on an LKB microtome and stained with a 1% mixture of azure and methylene blue.

Whole-Mount Antibody Staining. Embryos were fixed for either 1 hr at room temperature or overnight at 4°C in freshly prepared 4% (wt/vol) paraformaldehyde dissolved in 0.05 M sodium phosphate (pH 7.4) containing 4% sucrose and 0.15 mM $CaCl_2$. After two washes in buffer and two washes in 0.15 mM $CaCl_2$ in distilled water, embryos were permeabilized in −20°C acetone for 7 min and washed once with 0.15 mM $CaCl_2$ in distilled water and twice in 0.1 M phosphate-buffered saline containing 1% dimethyl sulfoxide and 1% bovine serum albumin (PDB solution). Nonspecific binding was blocked with 20% (vol/vol) normal goat serum in PDB solution. Embryos were incubated overnight in supernatant containing a monoclonal antibody (7A11) to which was added 1% dimethyl sulfoxide and 1% Triton. After three washes in PDB solution, embryos were incubated in 1:125 diluted peroxidase-conjugated anti-mouse IgG (Sigma), incubated with diaminobenzidine, and viewed as a whole mount.

RESULTS AND DISCUSSION

In initial experiments, nonstaged embryos were exposed to retinoic acid at concentrations of 1 or 0.1 μM for a period of 4 hr beginning at 10.5 hr of development (approximately the 3- to 6-somite stage). This treatment interval coincides with the formation of the primary optic primordia from the anterior region of the neural keel. In contrast to other vertebrates, the neural tube of teleost embryos develops as a solid mass of neuroepithelial cells, referred to as the neural keel. The optic primordia also form as a solid mass of cells from the anterior region of the neural keel. In embryos between the 7- and 9-somite stages (approximately 12–13.5 hr of development), an optic lumen arises within each primordium in parallel with the formation of the ventricles within the neural keel. As the optic lumen and ventricle enlarge and extend,

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they become continuous by way of the optic stalk by the 14-somite stage (≈16 hr).

Observations of embryos from the test groups, 15 hr after the end of retinoic acid exposure, showed a bifurcation of the eye usually along the horizontal meridian. Often pigmented cells were observed lining the bifurcation (Fig. 1*A*). Observations with Nomarski optics (Fig. 1*B*) or with conventional light microscopy (Fig. 1*C*) revealed the presence of two retinas in the bifurcated eyes. In embryos displaying this malformation, both eyes demonstrated retinal duplication (Fig. 1*C*). When 10.5-hr embryos were treated with 1 and 0.1 μM retinoic acid for 4-hr periods, the bifurcation was observed in all treatment groups, although the fraction of embryos displaying the malformation varied in a dose-dependent manner (Table 1). In animals not treated with retinoic acid, a bifurcated retina has never been seen. Increasing the time and/or concentration of retinoic acid exposure occasionally produced a more complicated phenotype. For example, embryos treated with 1 μM retinoic acid for 4 hr sometimes manifested more than one bifurcation.

The eyes in most cases were bisected symmetrically and two retinas of slightly reduced size, as compared with a single normal retina, had formed on either side of the bifurcation, one located dorsally and the other located ventrally (Fig. 1*C*). Occasionally one retina was substantially larger than the other. Variation was also seen in lens development within the

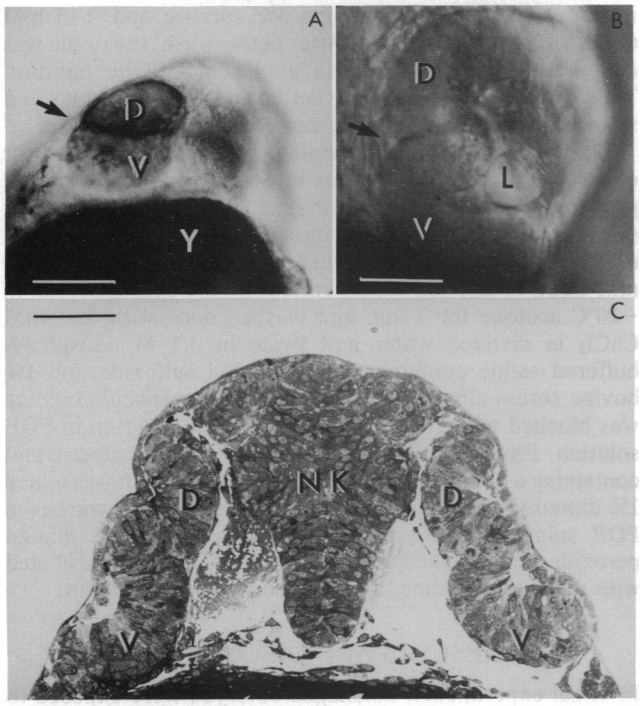

FIG. 1. (*A*) Bifurcated eye in a living zebrafish embryo viewed with a dissecting microscope. The bifurcation (arrow) divides the eye into dorsal (D) and ventral (V) regions. In this case, pigment cells line the bifurcation. The embryo was treated 10.5 hr after fertilization with 1 μM retinoic acid for 1 hr and viewed 15 hr later. Y, yolk. (Bar = 140 μm.) (*B*) Bifurcated eye viewed by Nomarski optics in a living zebrafish embryo. Two retinas are readily visualized; one is located dorsally (D) to the bifurcation (arrow); the other is positioned ventrally (V). A lens (L) is associated with the ventral retina. The embryo in this case was treated at a late gastrula stage with 1 μM retinoic acid for 1 hr and raised, thereafter, in 0.2 mM phenylthiocarbamide to suppress pigment formation. The photomicrograph was taken 30 hr after fertilization. (Bar = 95 μm.) (*C*) Conventional light micrograph of a section through the head region of a zebrafish embryo. Dorsal (D) and ventral (V) retinas are present on both sides of the neural keel (NK). (Bar = 120 μm.)

Table 1. Presence of bifurcated eyes

RA, μM	Treatment, hr	Eyes displaying bifurcation	
		No./total no.	%
0.1	1	29/195	14.8
	2	15/100	15.0
	3	33/100	33.0
	4	79/180	43.8
1	1	37/70	51.4
	2	79/110	71.8
	3	80/110	72.7
	4	117/185	63.2

Embryos were treated at 10.5 hr of development for 1–4 hr with retinoic acid (RA; 0.1 or 1 μM) and scored 15 hr later for the presence of bifurcated eyes.

malformed eyes, and eyes with none, one (Fig. 1*B*), or two lenses were observed.

The eye was not the only tissue affected by this treatment. The pace of development was slowed considerably throughout the embryo. Furthermore, forebrain tissue became truncated and the midbrain and hindbrain had an abnormal appearance. The otocyst, the peripheral auditory organ, was likewise altered, not only in its distance from the eye but also in its size and overall appearance. The tail, heart, and swimbladder all appeared abnormal as well. However, none of these, or other tissues within the zebrafish embryo, displayed a duplication or bifurcation in response to retinoic acid. Typically the animals died by 7 days of age.

Embryos were also treated with 10, 1, and 0.1 μM *all-trans*-retinol and *all-trans*-retinal at 10.5 hr of development for 4 hr. Retinol-treated embryos showed no eye malformations. Embryos treated with retinal at 1 and 0.1 μM also displayed normal eye development. Those embryos treated with 10 μM retinal showed some abnormal eye development, but duplication of the retina was not observed with this treatment. These data provide evidence that the retinoid causing duplication of the retina is retinoic acid itself.

Stage Specificity. To define more precisely the stages of zebrafish development during which exogenous retinoic acid induces retinal duplication, embryos were carefully staged and exposed to 1 μM retinoic acid for 1 hr. Embryos treated at 100% epiboly (the end of zebrafish gastrulation, ≈9.5 hr), tail bud stage (≈10 hr), 2–3 somites (≈10.5 hr), and 5–6 somites (≈11.5 hr) of development all displayed retinal duplication (Fig. 1*C*). Thus, the critical time period for this effect to be induced was 2 hr long when zebrafish embryos were incubated at 28°C. Embryos treated at earlier stages of gastrulation at 50% epiboly (≈5 hr) or 75% epiboly (≈8 hr) or at mid- and late-blastula stages (≈3–4 hr) lacked anterior head structures, including eyes. Similar effects of head truncation have also been observed in retinoic acid-treated *Xenopus* embryos when applied at similar development stages (9, 10). Embryos treated for 1 hr starting at the stages of 7–8, 10–12, 14–16, or 18–20 somites did not display the retinal duplication. However, embryos treated at the 19-somite stage (≈18.5 hr) or at 24 hr of development for 2.5 hr with 1 μM retinoic acid showed eyes of reduced size. A similar microphthalmia has been reported in *Xenopus* embryos treated with retinoic acid during neurulation (13).

The phenotype consisting of a single bifurcation with the formation of two symmetrical retinas (Fig. 1*B*) was most reliably observed when embryos were carefully staged by morphological criteria and treated at either the 100% epiboly or the 2- to 3-somite stage for 2 hr with 1 μM retinoic acid; in such experiments >95% of these embryos showed retinal duplication.

Cellular Analysis. To understand the cellular basis of the duplication phenomenon, eyecups of embryos treated at the 2- to 3-somite stage with 1 μM retinoic acid for 2 hr were sectioned at various times after treatment (Fig. 2). During normal zebrafish eye development, invagination of the optic primordia (occurring at the 14-somite stage, ≈16 hr) is accompanied by a flattening of pigment epithelial cells, progressing from the center to periphery of the eyecup. Mitotic cells are rarely observed within the pigment epithelial cell layer during this process. By the 17-somite stage (≈17.5 hr), the eyecup is well formed and the pigment epithelial cell layer across much of the eye has been reduced to a single layer of flattened cells that are closely apposed to the neural retina (Fig. 2*A*).

In treated embryos, the invagination of the eyecup is delayed and begins approximately at the 17-somite stage, at ≈19 hr or ≈2 hr later than in controls. Furthermore, whereas the pigment epithelium differentiates normally over the dorsal region of the eyecup as invagination begins, there is a striking proliferation in the pigment epithelial cell layer along the ventral border of the eyecup in the treated animals. Numerous mitotic cells are observed in this area. Cell proliferation continues in this ventral region during the 18- to 20-somite stage (20–22 hr; Fig. 2*B*) resulting in an expansion of the epithelium from a single layer of cuboidal cells to a pseudostratified epithelium three to four cell layers thick. By the 20- to 22-somite stage (21–22 hr), the expanded ventral epithelium resembles closely the dorsal retinal epithelium. The cells within the ventral epithelium are radially arranged in a manner similar, but at right angles, to those of the dorsal retinal epithelium. The ventral retinal epithelium remains separated from the dorsal retinal epithelium by the optic lumen (Fig. 2*C*).

Within an additional 2 hr (i.e., at 24 hr of development), the ventral retina is usually repositioned to assume an orientation similar to that of the dorsal retina (Fig. 2*D*). A single layer of flattened pigment epithelial cells lies over the distal surfaces of both retinas. Where the two retinas meet, they are often in direct contact along the vitreal border (i.e., along the ganglion cell layer) but are separated distally by the optic lumen. In some cases, pigment epithelial cells extend between the two retinas (Fig. 1*A*). Mitotic profiles are often observed along the midline border at these stages.

Laminae and major cell types are observed within both retinas by 2.5 days of development. In sections taken through

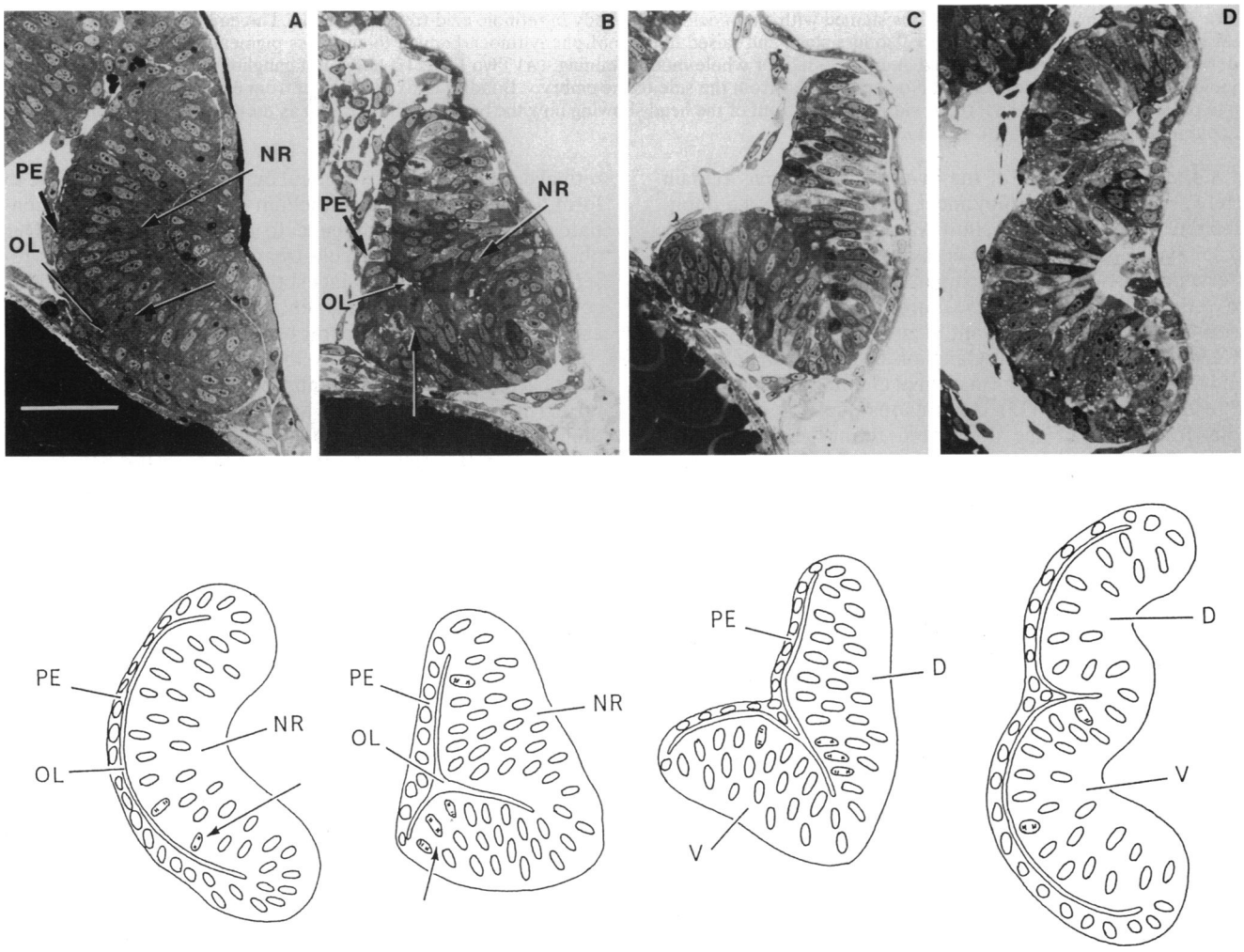

FIG. 2. Light micrographs of eye sections from control (*A*) and retinoic acid-treated (*B–D*) zebrafish embryos. The treated embryos were exposed to 1 μM retinoic acid at the 2- to 3-somite stage for 2 hr. Drawings illustrating the key features of the photomicrographs are shown below. (*A*) Control showing a normally developing eyecup at the 17-somite stage. The thin (and difficult to see) optic lumen separates the pigment epithelial cell layer (PE) from the neural retinal epithelium (NR). Some mitotic figures are seen in the neural retina (arrow). (*B*) In the retinoic acid-treated embryo at the 17-somite stage, a dramatic expansion of the epithelial cell layer in the ventral portion of the eyecup is seen. Several mitotic figures are observed in this region (arrow). (*C*) By the 22-somite stage of development in a retinoic acid-treated embryo, two retinal epithelial cell layers can be distinguished. The optic lumen separates the dorsal (D) and ventral (V) retinas and the neural retinas from the pigment epithelial cells (PE). (*D*) By 24 hr after fertilization, the ventral retina (V) has assumed the same orientation as the dorsal retina (D). The calibration bar in *A* is 40 μm and applies to all parts of the figure.

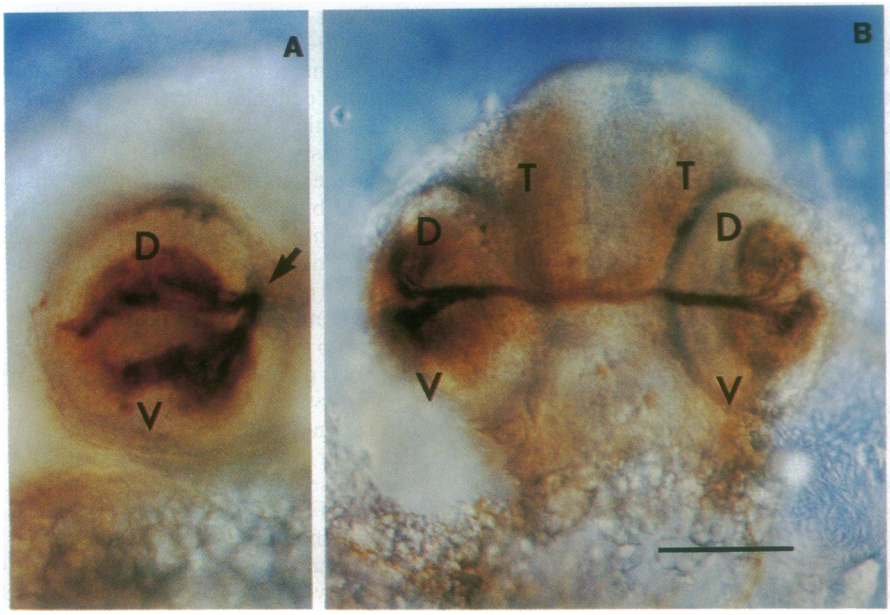

FIG. 3. Ganglion cells and their axons stained with a monoclonal antibody in retinoic acid-treated animals. The embryos were treated with 1 μM retinoic acid for 2 hr at the 2- to 3-somite stage and raised in 0.2 mM phenylthiocarbamide to suppress pigment formation. At 3.5 days of development, the embryo was fixed and processed for whole-mount staining. (*A*) Two fields (D and V) of ganglion cells can be seen in this retinoic acid-treated eye viewed with Nomarski optics from the side of the embryo. Bundles of axons project from each ganglion cell field and join to project centrally (arrow). (*B*) A view from the front of the head showing how the bundles of axons join as they exit the eye and innervate the contralateral tecta (T). (Bar = 170 μm.)

the most central regions of the eye, the two retinas remain largely separated where they meet at the midline; the duplicated retinas are continuous, however, along the vitreal border (i.e., along the ganglion cell layer) as noted above. Preliminary observations indicate some reorganization of cells within the two retinas along the midline border, reminiscent of that observed in the cyclops mutant in zebrafish (14).

Ganglion-Cell Projection. Ganglion-cell axons in zebrafish begin to grow out at ≈36 hr in normal animals and they reach the tectum by ≈60 hr (15). An obvious question is whether axons from both of the duplicated retinas extend to the tectum. Monoclonal antibody 7A11 selectively stains ganglion cells and their axons in developing zebrafish retinas and was used to study the distribution of the ganglion cells and the projection of their axons in retinoic acid-treated animals at 3.5 days of age. In most of the treated embryos, two distinct fields of ganglion cells were observed in each eye; each field corresponded to the ganglion cells of one retina (Fig. 3*A*). Bundles of axons from each ganglion cell field could be seen to project centrally. In some cases, however, axons from one field, or both, coursed within the eye and either never exited or exited at inappropriate locations. Occasionally, a third field of ganglion cells, located centrally, was observed. When two axon bundles exited the eye, the bundles joined as they left the eye (Fig. 3*B*), decussated at the chiasm, and innervated the contralateral tectum. It is not known whether all axons reach the tectum, what the topographic distribution of the axons on the tectum is, or whether inappropriate targets are innervated. An inappropriate innervation of ganglion-cell axons to the tectum has been observed in retinoic acid-induced microphthalmia in *Xenopus* embryos (9, 10).

In summary, we have observed that retinoic acid induces the formation of a second retina in zebrafish eyes exposed to this agent at a specific time during development. Furthermore, the second retina appears to be derived from cells in the ventral part of the developing eyecup that are normally destined to become pigment epithelial cells. In this regard, it is noteworthy that regeneration of the retina can occur in certain adult cold-blooded vertebrates from transdifferentiated cells of the retinal pigment epithelium (16–18). After retinal degeneration, the pigment epithelial cells proliferate to form a pseudostratified epithelium that eventually differentiates in an orderly sequence to form a new retina. Our observations on retinoic acid-treated zebrafish embryos resemble this process and suggest that retinoic acid can induce cells destined to become pigment epithelial cells to proliferate and differentiate into a neural retina.

Two enzymatic pathways that synthesize retinoic acid have been identified in the embryonic mouse retina (19) and, furthermore, these pathways are asymmetrically arranged in the dorso–ventral axis. In addition, evidence has been provided suggesting that retinoic acid levels in the developing mouse retina are among the highest in embryonic tissues. An obvious question is whether retinoic acid, or a related substance, is involved in retinal formation and differentiation. The retinoic acid-induced duplication of the zebrafish retina offers a system in which to study this question and the role of retinoids in pattern formation and cellular commitment during development.

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