Retinal stem cells and regeneration

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ABSTRACT **The optic vesicle gives rise to several very different epithelial tissues, including the neural retina, the pigmented epithelium, the iris, the ciliary epithelium of the ciliary body and the optic stalk. Retinal regeneration can arise from several different cellular sources; in some species, the process involves interconversion, or transdifferentiation, among cells of the different tissue types. Therefore, prior to a discussion of retinal regeneration, we will briefly discuss current knowledge about the influence of signaling molecules in cell fate determination in ocular tissues. Next, we will detail the evidence for neurogenesis in the mature retina. Lastly, we will describe various types of regenerative phenomena that occur in the retina, from complete regeneration of functional retina in fish and amphibians, to the more limited neuronal production that occurs in avian and mammalian retinas.**

KEY WORDS: *stem cell, progenitor, neurogenesis, optic, Müller*

Signaling molecules and the patterning of the optic vesicle

The initial evagination of the optic vesicle is followed relatively rapidly by a series of tissue changes: the vesicle invaginates into the optic cup; the pigment layer becomes considerably thinner; the neural retina retains a high rate of proliferation and thus thickens into a multicellular epithelium; and the rim of the optic cup maintains a lower rate of proliferation (see below), and develops into the monolayered epithelium of the iris and the ciliary epithelium of the ciliary body.

It has been known since the early part of the last century that the tissues surrounding the developing optic vesicle are critical for its development. For example, Holtfreter (1939) summed up the state of the literature, primarily from studies of amphibian embryos: "The naked eye primordium does not develop into tapetum (pigmented epithelium), multilayered retina, rods and cones, and it fails to fold inwards into an eye cup." In more recent years, better markers for the different domains of the optic vesicle have allowed us to understand better the nature of the inductive signals that regulate the patterning of the optic vesicle domains.

Direct evidence that the head mesenchyme is critical for the development of the pigment epithelium in chick embryos has only recently been uncovered (Fuhrmann *et al.*, 2000). By dissecting the optic vesicle free from the surrounding extraocular tissues, Fuhrmann and colleagues were able to demonstrate that the mesenchyme, but not the ectoderm, is required for expression of

genes specific to the pigmented epithelium (Fig. 1 B-D), including melanosomal matrix protein, and Micropthalmia Transcription factor (MiTF). In addition, the mesenchymal signal represses neural retinal specific genes, such as Chx10 (Fuhrmann *et al.*, 2000), indicating that this signal specifically directs the optic vesicle cells to the pigmented epithelial fate and suppresses the neural retinal precursor fate.

Investigations into the molecular nature of the pigment epithelial promoting factor indicated that a member of the TGF-beta superfamily was likely involved. Activin, but not several other BMP-like proteins, could replace the mesenchyme and induce nearly 100 percent of the optic vesicles to develop pigmented epithelium (Fuhrmann *et al.*, 2000). Although the specific activin-like molecule produced by the mesenchyme has not yet been identified, Adler and Belecky-Adams (2002) have found that overexpression of noggin, a BMP inhibitor, leads to replacement of the ventral RPE by neuroepithelium-like tissue, consistent with a role for an endogeneous BMP/activin-like signal.

In addition to this BMP/activin signal, another class of signaling molecules has also been implicated in the development of the pigmented epithelium. Members of the hedgehog gene family are expressed in the developing eye. Indian hedgehog and desert hedgehog are expressed in the pigmented epithelium, while Sonic

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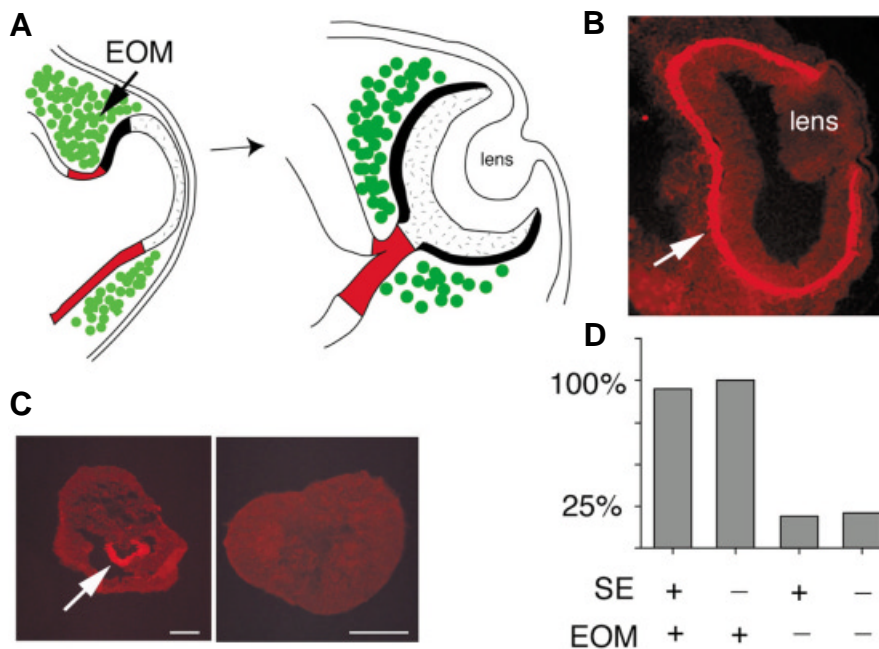
Fig. 1. **(A)** *Development of the optic cup from the optic vesicle requires interactions with surrounding mesenchymal cells (green) to promote development of the pigmented epithelium (black), while overlying lens ectoderm promotes neural retinal formation (speckled). Optic stalk (red) requires Shh signaling from the midline of the diencephalon. (B,C).* In vivo **(B)** *and* in vitro **(C)** *(left panel) development of the melanosomal matrix protein (immunolabeling) in the pigmented epithelium (arrows) requires interaction with extraocular mesenchyme; removal of this tissue inhibits expression of this gene (C, right, and D) (from Fuhrmann* et al., *2000).* **(D)** *Percentage of explants with RPE formation in the presence (+) or absesnce (-) of the surface ectoderm (SE) or extraocular mesenchyme (EOM).*

hedgehog is expressed in the optic stalk and retinal ganglion cells (Levine *et al.*, 1997; Wallace and Jensen, 1997; see Hartenstein and Reh, 2000, for review). Inhibition of the hedgehog pathway using cyclopamine treatment in amphibian embryos results in defects in pigmented epithelial differentiation (Peron *et al.*, 2003), while overexpression of Shh also affects pigmented epithelial development (Zhang and Yang, 2001). However, mice deficient in Ihh and/ or Shh in the retinal ganglion cells do not show the same defects in pigment epithelial development (Dakubo *et al.*, 2003). Therefore, there may be functional redundancy with other hedgehog family members, or, alternatively, the hedgehog pathway may be partly redundant with the BMP/activin pathway in pigment epithelial differentiation.

A further link between hedgehog and RPE development comes from recent data indicating a connection between Shh signaling and Gas1, a GPI-linked membrane protein and member of the "growth arrest specific" factor family. Deletion of Gas1 in mice results in a failure of RPE differentiation and a change in fate of the presumptive RPE to neural retina (Lee *et al.*, 2001a). Gas1 interacts with hedgehog through direct binding, and can act either as an inhibitor to the Shh pathway (e.g., in somites, Lee *et al.*, 2001b) or together with Shh as a growth inducer. While the specifics of the interaction in RPE development are not clear, future studies of the interactions between these signaling pathways are warranted (Mullor and Ruiz i Altaba, 2002).

While the developing pigmented epithelium lies immediately adjacent to the extraocular mesenchyme, the neural retina forms from the distal-most part of the optic vesicle, in close apposition to the ectoderm. Several lines of evidence indicate that it is not merely the isolation of this part of the optic vesicle from the pigmented epithelial inducer in the mesenchyme that allows the distal optic vesicle to form retina. Rather, there is an active signaling relationship between the ectoderm and the optic vesicle that promotes the neural retina fate. As noted above, it has been known from Holtfreter's time that the isolated optic vesicle will not develop into neural retina.

While some markers of retina, such as Chx10, continue to be expressed in the isolated vesicle (Fuhrmann *et al.*, 2000), this tissue does not continue its development. In addition, removal of just the ectoderm from the vesicle causes severe defects in eye development, including loss of the neural retina, though some pigmented epithelial tissue develops (Hyer *et al.*, 1998). An early clue that the formation of the neural retina is not merely the "default" state of the optic vesicle came from transplantation studies. When the optic vesicle is transplanted to ectopic sites in the embryo, the presumptive pigmented epithelial layer can develop instead as neural retina (Fig. 2A).

The molecular nature of the neural retina "inducer" likely involves one or more of the FGF family of peptides. Pittack *et al.* (1997) found that treatment of chick optic vesicle cultures with FGF causes the expansion of the neural retina domain. In some cases, the entire presumptive pigment epithelial layer develops instead as retina (Fig. 2 B,C). (See also Hyer *et al.*, 1998 and Vogel-Hopker *et al.*, 2000.) Similar studies have been performed in the mammal (Nguyen and Arnheiter, 2000; Zhao *et al.*, 2001), and thus the sufficiency of FGF for neural retinal fate is likely conserved across the vertebrate lineage. Activation of the MAP kinase signaling pathway is also sufficient to convert presumptive RPE to neural retina, again consistent with a receptor tyrosine kinase activation being sufficient to direct the optic vesicle cells to a neural retinal fate (Zhao *et al.*, 2001; Galy *et al.*, 2002). FGF likely functions both to stimulate proliferation and to specifically down-regulate RPE-specific gene expression (Fuhrmann *et al.*, 2000; Nguyen and Arnheiter, 2000).

Since several different FGFs are expressed in the developing eye—in the presumptive lens ectoderm and the optic stalk, as well as in the developing ciliary epithelium—it is likely that some redundancy exists in this system (see de Iongh and McAvoy, 1993; McCabe *et al.*, 1999, for review; Vogel-Hopker *et al.*, 2000). Thus, it has been difficult to demonstrate a requirement for FGF in the development of the neural retinal fate. However, blocking FGF receptors prevents neural retina development, but does not inter-

fere with the development of the RPE (Pittack *et al.*, 1997). Overall, the data from many different labs, in several different species, support the following model of signaling systems in eye development (Fig. 2D; Fuhrmann *et al.*, 2000). FGFs, derived from the optic stalk, the lens ectoderm, or the vesicle cells themselves, promote/maintain the neural retinal fate in the distal part of the optic vesicle, either through specifically activating eye transcription factors (Martinez-Morales *et al.*, 2001) or by maintaining a high level of proliferation, or both. An activin/BMP-like molecule, produced by the extraocular mesenchyme, antagonizes the FGF signal and alternatively promotes the pigmented epithelial fate by activating RPE-specific genes, like MiTF, through the suppression of neural retina-specific genes, like Chx10, and/or by inhibiting the proliferation of the presumptive RPE cells.

Other highly specialized epithelial tissues develop from the optic cup. The ciliary epithelium and iris develop from the rim of the optic cup, in close proximity with the lens. The ciliary epithelium has several important ocular functions, including secretion of aqueous humor and both the synthesis and attachment of the suspensory zonule fibers that support the lens. While morphological studies have shown that this region of the eye becomes morphologically distinct some time after optic cup formation (see Beebe,1986 or Kubota *et al.*, 2004, for review), recent studies using molecular markers for ciliary epithelial gene expression have shown that this region of the optic cup acquires a distinct pattern of gene expression prior to signs of morphological differentiation. Kubota *et al.* (2004) used a subtractive library approach to identify a large number of ciliary epithelium-specific genes. Many of these are expressed very early in eye development and maintain their ciliary epithelial pattern into adulthood (Fig. 3 A,B,C,E). At approximately the same time in development that the ciliary epithelial pattern of gene expression is established, the rate of mitotic activity in this region of the optic cup begins to diverge from the rate in the presumptive neural retina (Fig. 3 D,E). The retina shows steady increases in the density of mitotic cells, while the ciliary epithelium maintains only a very low rate of proliferation. This results in a large difference in the rate of proliferation between the developing ciliary epithelium and the retina proper, and the ultimate result is that the ciliary epithelium remains a monolayer, while the retina develops many layers, and many more cells overall.

The molecular mechanisms that define the domain of the ciliary epithelium, as distinct from the neural retina, are not well understood. Both BMP and Wnt signaling are active in the presumptive ciliary epithelium and iris. BMP4, BMP2, and BMP7 are all expressed in the developing ciliary epithelium (Papalopulu and Kintner, 1996; Furuta and Hogan, 1998; Belecky-Adams and Adler, 2001; Trousse *et al.*, 2001; Zhao *et al.*, 2002; Liu *et al.*, 2003). Msx1 and Msx2, thought to be direct targets of BMP signaling, are also expressed in this region (Foerst-Potts and Sadler, 1997), suggesting that there is an autocrine BMP signal critical for ciliary epithelial development and that forced expression of Msx2 suppresses proliferation in the neural retinal epithelium. Moreover, antagonism of BMP signaling in this region of the eye disrupts the normal development of the ciliary epithelium (Zhao *et al.*, 2002), and mutations in BMP4 cause ciliary epithelial and/or iris defects in mice (Chang *et al.*, 2001). Despite this emerging body of evidence supporting BMP involvement in ciliary epithelial development, there is also evidence for a role for the Wnt signaling pathway in patterning this domain of the eye. Transgenic zebrafish with a

reporter construct for Wnt pathway activation (Lef1) show a high level of activity in the ciliary epithelium and iris (Dorsky *et al.*, 2002). In addition, Lef1 expression (Kubo *et al.*, 2003), a transcriptional target of Wnt signaling, is coincident with the domain of developing ciliary epithelium. A prime candidate for a role in the regulation of ciliary epithelial regulation is Wnt13/2b. This gene is expressed in
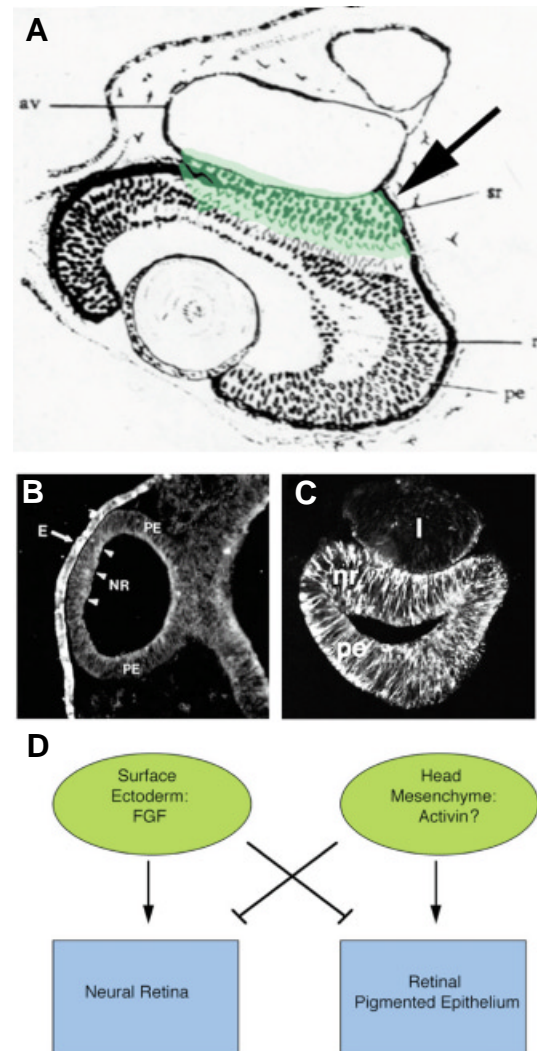
Fig. 2. Development of the RPE. (A) *Transplantation of optic vesicle to ectopic locations in frog embryos results in the transformation of the pigmented epithelium into neural retina (arrow pointing to green shaded region; from Holtfreter).* **(B)** *FGF-2 is expressed in the presumptive lens ectoderm (E) of the chick embryo overlying the optic vesicle (NR, presumptive neural retina; PE, presumptive pigmented epithelium).* **(C)** *If an excess of FGF is present in the optic vesicle, a retinal duplication occurs and the presumptive pigmented epithelium (pe) begins to express Neurofilament protein, which is normally expressed only in the neural retina (from Pittack* et al.*, 1997; l, lens).* **(D)** *The model of optic vesicle patterning by signaling factors derived from these studies is that an activin/BMP-like molecule secreted by the extraocular mesenchyme causes the adjacent proximal part of the optic vesicle to develop as pigmented epithelium, while FGF, from the overlying lens ectoderm and/or from within the optic vesicle itself, antagonizes the activin/BMP signal and allows/promotes neural retinal development in the distal part of the optic vesicle (from Fuhrmann* et al., 2000).*
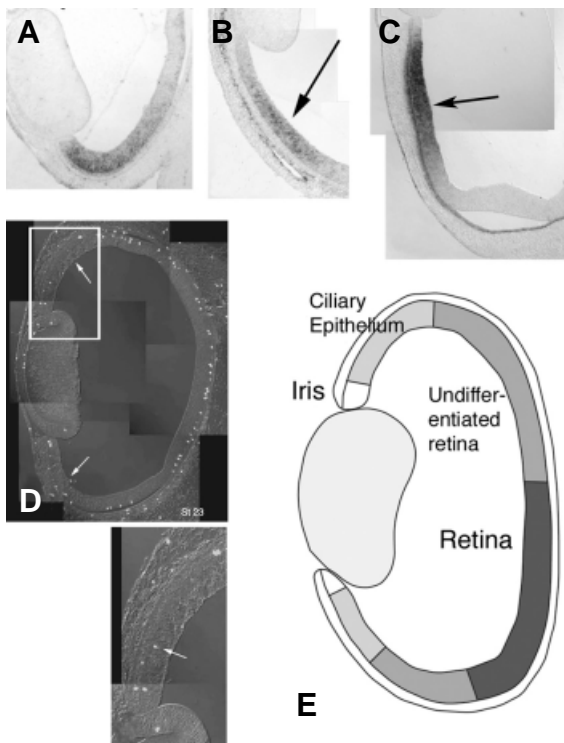
Fig. 3. Development of the ciliary body. (A,B,C) In situ *hybridization at Stage 23/24 of chick eye showing gene expression of three ciliary epithelium-specific genes, indicating that this part of the optic cup is distinguished from the rest of the retinal epithelium very early in development.* **(D)** *Labeling mitotic figures with phosphohistone H3 shows that, as early as stage 25, there are significantly fewer mitotic figures in the presumptive ciliary epithelium than in the presumptive retinal epithelium in more central regions.* **(E)** *Model of eye development summarizing the patterning of the epithelium into iris, ciliary epithelium, and retina, both differentiated and undifferentiated, to show the region from which the CMZ will ultimately arise (from Kubota* et al., *2004).*

present at the end of embryogenesis (Fig. 4). In the goldfish eye, Johns has estimated that the CMZ adds up to 12,000 new neurons per day. In frogs, the size of the CMZ and production of new neurons is substantially reduced after metamorphosis. However, the CMZ of fish continues to generate new retinal neurons throughout life, and, accordingly, this region of stem cells persists. Using lineage-tracing dyes injected directly into cells at the retinal margin, Wetts *et al.* (1989) have shown that these cells give rise to clones of assorted sizes, ranging from one to 104 cells. These clones are composed of retinal cells of various morphologies and laminar positions, including pigment epithelium, strongly suggesting the totipotency of progenitors at the retinal margin (Wetts *et al.*, 1989). Perron *et al.* (1998) have demonstrated that the CMZ represents a spatial distribution of retinal development, with the most primordial cells located the most peripherally and the cells with increasing specificity located toward more central regions.

In contrast to fish and amphibians, avian retinal histogenesis takes place nearly exclusively during embryogenesis. In the chicken,

the ciliary epithelium (Zakin *et al.*, 1998); overexpression suppresses neural retinal development and upregulates at least one ciliary epithelial gene: Collagen IX (Kubo *et al.*, 2003). The relationship between BMP and Wnt signaling remains to be understood, as do their relative contributions to the regulation of both ciliary epithelial and cell cycle gene expression.
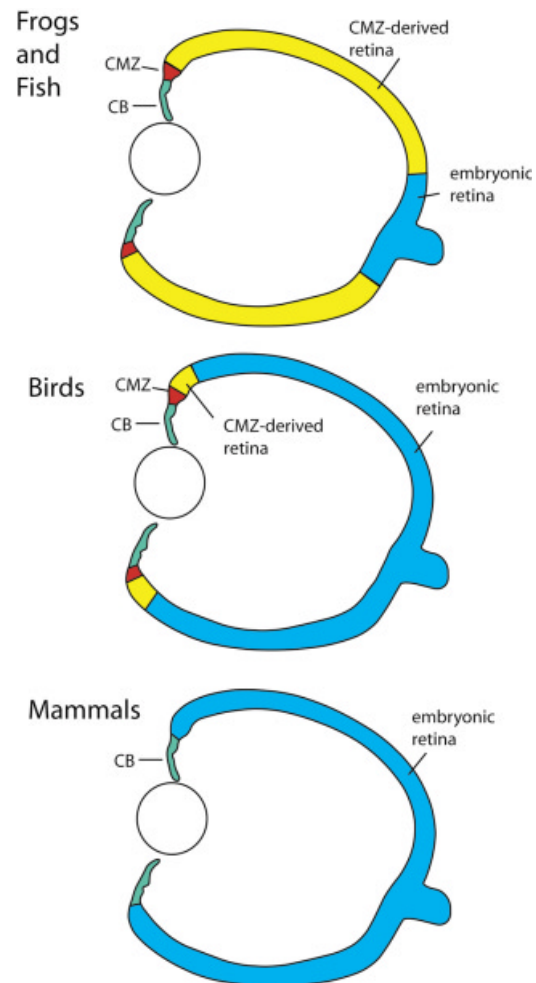
Retinal stem cells and the ciliary marginal growth zone

It has been known for many years that, in fish and amphibians, only the most central part of the retina is formed during embryogenesis. Most of the retina in these animals is formed by the addition of new retinal neurons from a growth zone at the retinal margin, known as the CMZ (ciliary margin zone). The addition of new neurons at the CMZ in amphibians was first directly demonstrated by (^{3}H)-thymidine labeling studies of Hollyfield and of Gaze and colleagues (Hollyfield, 1968; Straznicky and Gaze, 1971; Gaze and Watson, 1968). Peripheral regions of the retina in these animals grow with the addition of concentric rings of new cells that are generated by a zone of stem cells at the retinal margin.

These CMZ cells must be either self-renewing or capable of generating an enormous number of progeny, since the total number of retinal cells generated far exceeds the number of CMZ cells

Fig. 4. The relative contribution of the CMZ to retinal growth has been progressively reduced in homeothermic vertebrates and is shown in drawings of adult eyes. *The region of retina generated in the embryonic/ neonatal period is shown in blue, while that generated by the CMZ is shown in yellow for the various vertebrate classes. The CMZ itself is indicated in red to show the relationship to the ciliary body (CB) colored green.*

the vast majority of cells within the retina are generated in about 10 days of embryonic development (Fujita and Horri, 1963; Prada *et al.*, 1991). Throughout the process of neurogenesis in the retina, the epithelium is composed of both mitotically active precursor/ stem cells and their postmitotic neuronal progeny. The generation and differentiation of all retinal cell types begins in central retina and proceeds to peripheral regions (Prada *et al.*, 1991; McCabe *et al.*, 1999). Willbold and Layer (1992) have demonstrated that peripheral regions of the developing chick retina (up to E9) retain the ability to produce all types of retinal neurons *in vitro*. At the time of hatching, about E21, functional retinal circuitry has been established and the chicks are able to see.

Although a study by Morris *et al.* (1976) demonstrated that some cells could be labeled with (H3)-thymidine in the far peripheral retina of the post-hatch chick, only recently has it been shown that the posthatch bird retina has ongoing neurogenesis at the retinal margin similar to that in lower vertebrates (Fischer and Reh, 2000). The source of new cells added to the chick retinal margin is similar to the source for amphibians and fish: a group of neural progenitor cells located immediately adjacent to the peripheral edge of the retina (Fig. 5 A-D). These cells can be identified by their expression of proteins normally present in embryonic retinal progenitor cells, including Chx10, Pax6, Cash1, Cath5, Hairy1, and cell cycle proteins (Fischer and Reh, 2003, and unpublished observations). The CMZ cells of the chicken generate new retinal neurons that are incorporated into the existing retina. Immunohistochemistry for BrdU and for antibodies specific to particular types of retinal cells revealed that amacrine, bipolar, and Müller cells are the predominant cell types generated in the posthatch chicken (Fig. 5 B-D). In the untreated posthatch chick eye, there is no evidence that either photoreceptors or ganglion cells continue to be generated, although certain treatments can stimulate ganglion cell genesis at the CMZ (see below).

One apparent difference between the CMZ of posthatch chickens and that of lower vertebrates is that the posthatch chick CMZ normally only generates a subset of retinal neurons, rather than the full complement. However, this restriction appears to be due to the lack of a microenvironment conducive to ganglion cell production, rather than to an intrinsic limitation on the cells. Intraocular injections of insulin and FGF-2 induce the production of ganglion cells from the CMZ, as indicated by the presence of cells labeled for BrdU and Islet-1, Neurofilament, RA4, and Brn3 (Fischer *et al.*, 2002; Fig. 5 E,G,H). In addition, while Cath5, a critical determinant of ganglion cell fate, is not normally expressed by the cells of the CMZ, the intraocular injections of FGF and insulin induced the expression of this transcription factor in the CMZ (Fig. 5F). Thus, the CMZ cells of the chicken retina are not intrinsically limited to the production of only a few cell types. Microenvironment permitting, they may have full potential to generate all types of retinal neurons.

Studies are now unfolding the molecular mechanisms that regulate proliferation of progenitors and stem cells at the retinal margin. Many of the factors that control neurogenesis in the developing retina and central nervous system are also mitogens in the CMZ. Studies in goldfish—using autoradiography with IGF-1 tagged with iodine-125—have shown specific binding of this growth factor at the retinal margin (Boucher and Hitchcock, 1998b). Also, intraperitoneal injection of growth hormone increases proliferation of retinal progenitors at the CMZ (Boucher and Hitchcock, 1998a; Otteson *et al.*, 2002). Intraocular injections of mitogenic factors can stimulate the production of neurons in the CMZ of birds. Both

insulin and IGF-I act as mitogens in the chick and quail CMZ (Fischer and Reh, 2000; Kubota *et al.*, 2002), suggesting that fish and birds share at least some regulatory mechanisms for CMZ cells. One difference between these species is that EGF can
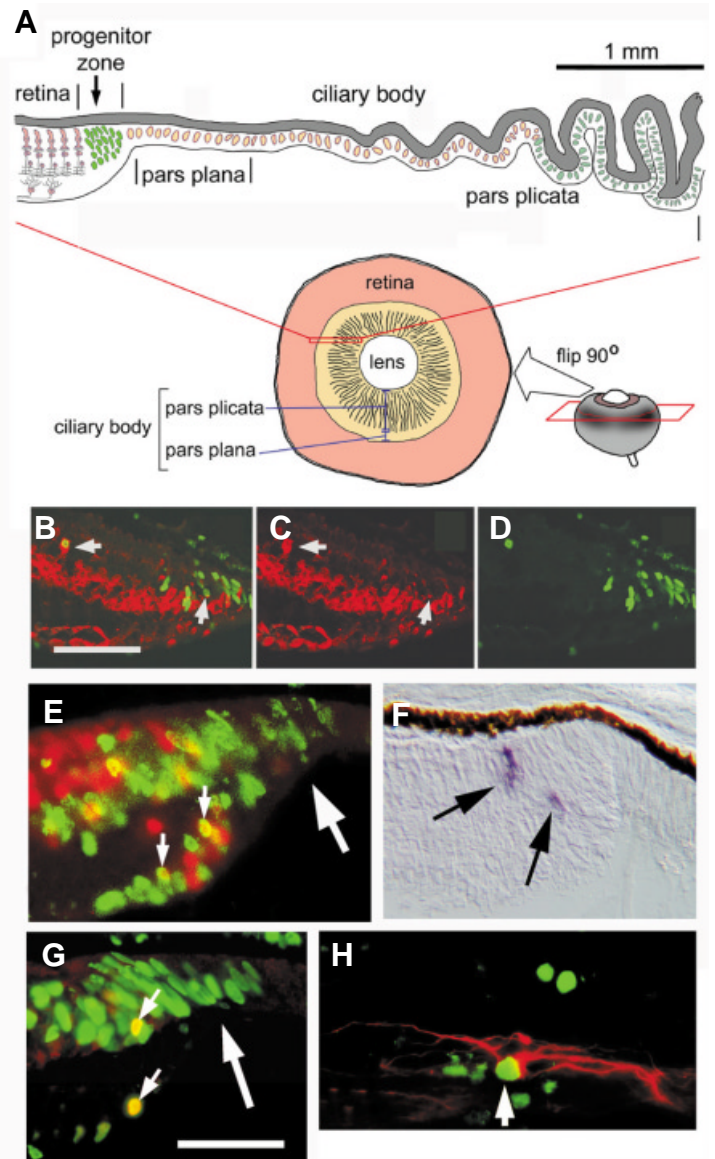
Fig. 5. The ciliary marginal zone of the chicken eye. (A) *The organization of the anterior of the chicken eye, showing the relationship between the two parts of the ciliary body -the pars plana and the pars plicata- and the progenitor zone or CMZ (from Fischer and Reh, 2002). (B,C,D). In panels A-H, the vitreal surface of the retina is towards the bottom of the panel. Double-labeled immunohistochemistry for Hu* **(C)**, *(a protein present in amacrine cells) and BrdU* **(D)**, *and merged* **(B)** *to show generation of new neurons (arrows) at the CMZ of the posthatch chicken (from Fischer and Reh, 2000). E-H. Ganglion cells are generated by CMZ cells in the posthatch chicken, following intraocular injections of FGF-2 and insulin.* **(E)** *Double-labeled immunohistochemistry for BrdU (green) and Islet-1 (red). Small arrows point out double-labeled cells.* **(F)** *Cath5 in* situ *hybridization at the CMZ (labeled cells are pointed out by arrows).* **(G)** *Double-labeled immunohistochemistry for BrdU (green) and Brn3 (red); small arrows point to newly generated ganglion cells.* **(H)** *Double-labeled immunohistochemistry for Neurofilament and BrdU; arrow points out newly generated ganglion cell. (E-H from Fischer et al., 2002.)*
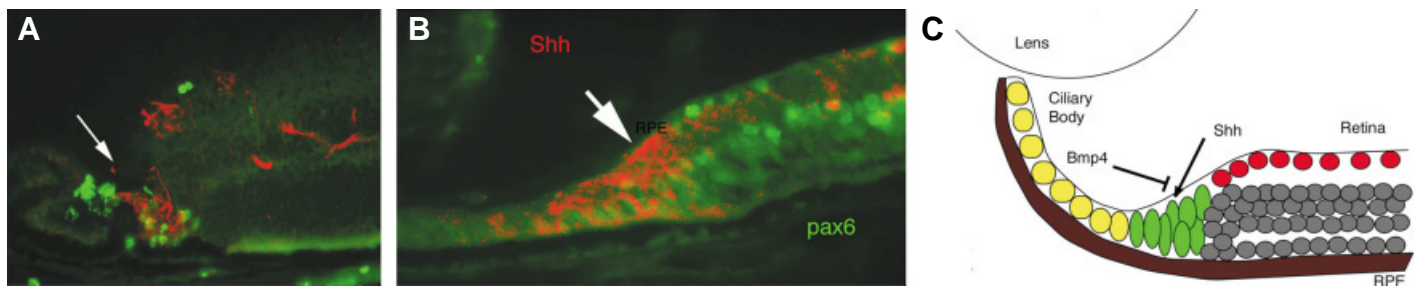
Fig. 6. Shh and the CMZ in birds and mammals. (A) *Section through the retinal margin of a two-week old mouse that has a mutation in one allele of the Shh receptor ptc. The animal has received injections of BrdU to label S-phase proliferating cells, and a small cluster of these cells is found at the retinal margin of these mutant animals (green). The cells are also labeled with the progenitor marker nestin (red) (from Moshiri and Reh, 2004). These are not present in normal mice.* **(B)** *In posthatch chicks, Shh (red) is concentrated at the CMZ, and some of the protein is associated with Pax6 (green)-labeled ganglion and amacrine cells (Moshiri and Reh, in press).* **(C)** *A model of CMZ: regulated by Shh derived from the ganglion cells, and antagonized by BMP derived from the ciliary epithelium.*

stimulate CMZ proliferation in the posthatch chick (Fischer and Reh, 2000), but not in the fish (Boucher and Hitchcock, 1998a). More recently, we have examined another class of growth factors, the hedgehog family of signaling molecules, for their potential role in regulating neurogenesis at the retinal margin. We have found that Sonic hedgehog (Shh) is concentrated at the CMZ of posthatch chicks (Fig. 6B) and that intraocular injections of an Shh-signaling inhibitor, cyclopamine, inhibits neuronal proliferation *in vivo* (unpublished observations, A. Moshiri and T.A. Reh). Thus, there are at least three mitogenic factors that regulate neurogenesis at the retinal margin. As additional studies of this region emerge, it is likely that even more factors will be identified.

The mammalian retina also follows the general pattern of central to peripheral growth and neurogenesis, and the last mitotically active progenitor cells in the mammalian retina are near the retinal margin. However, once retinal histogenesis is complete in the mammal, there is no continued production of retinal neurons, and there is no evidence for a CMZ-like growth zone. In normal mice, retinal histogenesis is complete in the first postnatal week, and BrdU-labeling studies after this time fail to show S-phase cells at the retinal margin (Kubota *et al.*, 2001). Moshiri and Reh (2003) delivered injections of BrdU every two hours from postnatal day 14 to postnatal day 16, and found only scattered labeled cells within the ciliary epithelium, and very rarely a labeled cell at the retinal margin. In older animals, even

this low level of labeling was absent. In the primate retina, similar results were obtained (Fischer, Hendrickson, and Reh, unpublished observations), and injections of BrdU in monkeys older than one year of age failed to demonstrate a CMZ-like zone at the retinal margin. Studies in adult human retina have closely examined the expression of the neural progenitor marker nestin in the retina. Nestin is expressed in some cells at the junction of the neural retina and the ciliary body, which suggests that a remnant of the CMZ may remain in humans (Mayer *et al.*, 2003). Thus, it appears that the CMZ has made a progressively smaller contribution to the growth of the retina in the evolution of higher vertebrates (Fig. 4).

Nevertheless, there have been some reports that cells isolated from the ciliary epithelial region or retinal margin of mice can give rise to "neurospheres" *in vitro* (Tropepe *et al.*, 2000; Ahmad *et al.*, 2000). These have been termed "retinal stem cells," though their relationship to the CMZ of other vertebrates is not clear. The neurosphere-generating cells are initially pigmented, and therefore are most likely from the iris or pigmented epithelial layer of the ciliary epithelium. Their ability to generate neurons may therefore be more akin to the transdifferentiation phenomenon that underlies retinal regeneration in amphibians and embryonic chicks, and will be discussed later in this chapter.

Despite the absence of a CMZ in the mammalian retina, there is some evidence that this region of the eye still contains potential
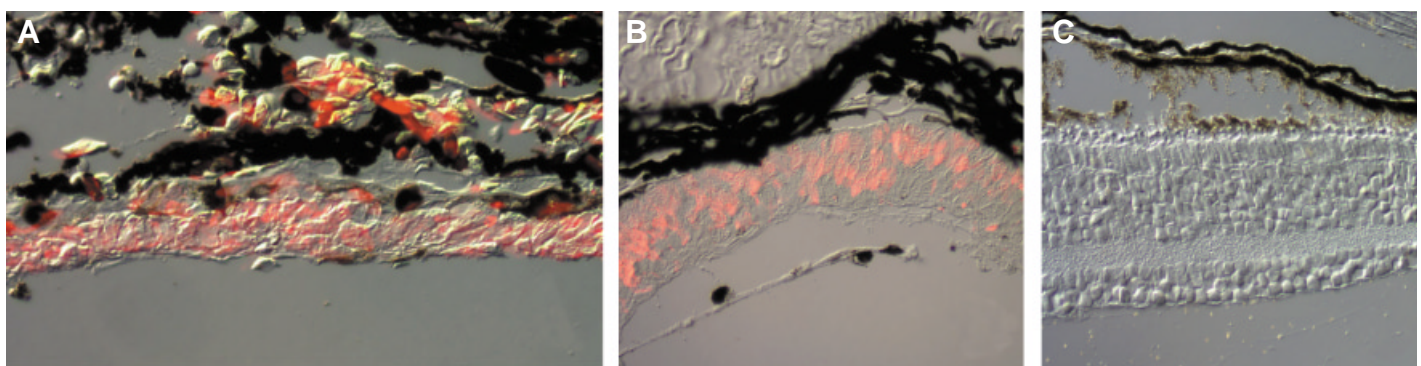
Fig. 7. Regenerating newt retina, showing the re-growth of the retina from the pigmented epithelium. *Two weeks after retina removal, a layer of pigmented cells has lost pigmentation and expresses PCNA (red in* **(A)***). By 3 weeks, these cells are actively dividing (PCNA in* **(B)***) and by 5 weeks, they have finished proliferation and have formed a new, laminated retina* **(C)**. *(Courtesy of Sanae Sakami.)*

for neurogenesis. In an examination of mice with mutations in signaling pathways, we discovered that mice with a single allele of the Shh co-receptor gene, patched (ptc), have a small zone of proliferating cells at the retinal margin that is highly reminiscent of the CMZ of other vertebrates (Moshiri and Reh, 2004; Fig. 6A). Injecting these animals with BrdU (with the same schedule as described above for normal mice) reveals many labeled cells at the retinal margin. These BrdU-labeled cells express markers of retinal progenitors, such as Chx10, Pax6, and nestin. When animals are allowed to survive for several days after the BrdU administration, some of the labeled cells are incorporated into the retina and express antigens of retinal neurons. Thus, while the normal mouse retina does not possess a distinct CMZ, partial activation of the Shh pathway enables such a zone to form (Fig. 6C). Why does the ptc+/- mouse retina show a CMZ like region? We suspect that the mammalian retina has lost the Shh at the peripheral margin necessary to maintain the CMZ in other vertebrates. Indeed, in normal mice we do not see a concentration of Shh at the peripheral margin of the retina like we do in the chick (see above).
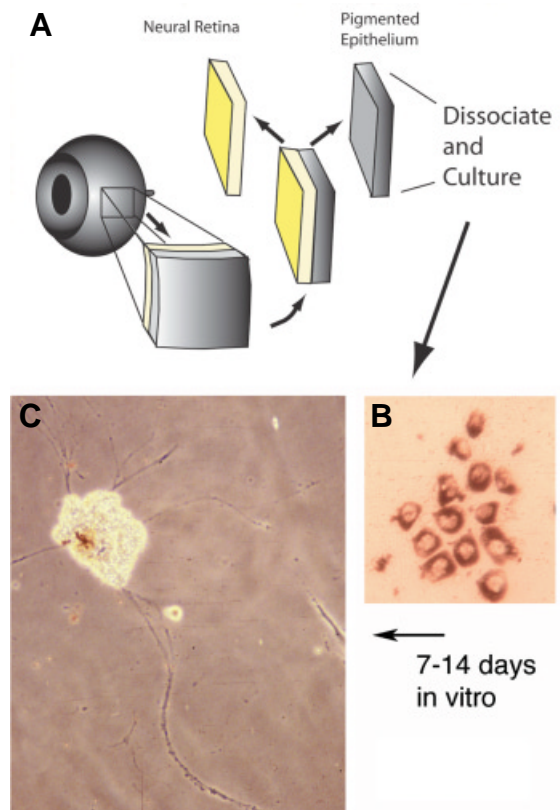
Regeneration of retina from the pigmented epithelium

The 18th-century studies of Charles Bonnet revealed the remarkable regenerative powers of the amphibian eye. The amphibian ability to regenerate retina from the pigmented epithelium has been most extensively studied in urodele amphibians, particularly the newt and axolotl, though anuran (frog) tadpoles are also able to regenerate retina from this source. The most typical experimental design is to remove the retina, leaving the pigmented epithelium intact. The pigmented epithelium then loses pigmentation, proliferates and generates two new epithelial layers: a pigmented layer and a non-pigmented layer (Fig. 7). The non-pigmented layer begins to express genes typical of retinal progenitor cells and undergoes extensive proliferation, generating enough cells for an entirely new retina. The process of regeneration is thus a two-step process. The first step is the dedifferentiation of the pigmented epithelium into retinal progenitors, and the second step is much like normal development of the retina: the progenitor cells proliferate and then differentiate into the various retinal cell types.

Evidence that the pigmented epithelium dedifferentiation is the source of new retina comes from two types of experiments. Stone, Loposhov, and others have transplanted pieces of pigmented epithelium from one animal to the posterior chamber of the eye of another animal. (See Reh and Levine, 1998, for review.) Within a few weeks, the piece of pigmented epithelium transforms into multilayered neural retina. *In vitro* experiments have also been used to confirm the pigmented epithelial source of neural retinal tissue. Isolated pigmented epithelial cells can be cultured and, under particular culture conditions, can dedifferentiate *in vitro* and generate new retinal neurons (Fig. 8; Reh *et al*., 1987). The demonstration of the dedifferentiation of pigmented epithelial cells has been facilitated by the fact that these cells have an intrinsic marker: their pigmentation. The regeneration of retina from the pigmented epithelium has thus been one of the first demonstrations of "transdifferentiation" (Okada, 1981).

A similar phenomenon of pigmented epithelial transdifferentiation also occurs in embryonic chick eye (Coulombre and Coulombre,

1965; Reh and Pittack, 1991; Park and Hollenberg, 1989). If the neural retina is removed from the chick embryo eye within the first three to four days of incubation, the pigmented epithelium of the chick can undergo a transdifferentiation into neural retinal progenitors, similar to that in the amphibian. The neural retinal progenitor cells are organized like those of the normal retina. However, they retain the polarity of the pigmented epithelium and therefore give rise to an "inverted" retina. Thus, one clear difference between the chick embryo and the amphibian is the failure of the chick's pigmented epithelial cells to detach from the underlying basement membrane during the regeneration process. Nevertheless, the retina that forms following pigmented epithelial transdifferentiation is laminated like normal retina, and contains relatively normal rations of the different retinal cell types.

The molecular mechanisms underlying retinal regeneration from the pigmented epithelium are only beginning to be understood, though much of the process appears to mirror aspects of normal development. As noted above, tissue interactions regulate the development of the different tissue types from the optic vesicle. Signaling molecules, including FGFs, BMPs, and hedgehogs, are thought to mediate these tissue interactions. Similar signaling molecules may be critical for the transdifferentiation process. Coulombre first noted that the transdifferentiation of the pigmented epithelium in chick embryos required that a small amount of neural retinal tissue remain in the eye. In Xenopus tadpoles and chick embryos, FGFs can replace the piece of neural retina and stimu-

Fig. 8. Regeneration of RPE *in vitro*. *Amphibian pigmented epithelium grown in dissociated cell culture generates small clusters of neural cells that extend extensive axonal processes after two weeks* in vitro *(from Reh* et al*., 1987).*

late the process of regeneration from the pigmented epithelium alone. FGF-1, FGF-2, or FGF-8 can all induce retinogenesis in the pigmented epithelium (Park and Hollenberg, 1991; Pittack *et al.*, 1991, 1997). By contrast, activin can block retinal regeneration from the pigmented epithelium, even in the presence of FGF (Pittack and Reh, unpublished observations). Thus, at least these two developmentally important signals are also active in regulating the transition between pigmented epithelium and retinal precursors during regeneration.

Regeneration of retina from the CMZ

In addition to adding retinal area during normal eye growth, the CMZ is also capable of regenerating retina after damage. The CMZ of fish continues to produce retinal neurons throughout the life of the animal and can also replace damaged retinal cells in the peripheral retina. For over a quarter of a century, it has been known that retinal injury in fish causes increased proliferation at the CMZ, and this finding has been confirmed in various lesion paradigms (Lombardo, 1968, 1972; Maier and Wolburg, 1979; Kurz-Isler and Wolburg, 1982; Raymond *et al.*, 1988; Hitchcock *et al.*, 1992). For example, Neurotoxic doses of 6-hydroxydopamine increase the width of the CMZ in fish by 50 percent (Negishi *et al.*, 1987). However, the regenerated tissue from the CMZ after neurotoxic lesions in adult goldfish lacks the proper organization of cells seen in normal retina (Stenkamp *et al.*, 2001).

The CMZ of the amphibian retina increases its proliferative rate in response to surgical lesions (Gaze and Watson, 1968; Keefe, 1973; Reh and Nagy, 1987). Retinal damage resulting from transient ischemia in newts results in a ten-fold increase in (3H)-thymidine incorporating cells at the CMZ (Keefe, 1973), and similar responses occur in frog tadpoles in response to ischemic injury (Reh and Nagy, 1987). Neurotoxic damage to the retina can also induce proliferation at the CMZ. Kainic acid-mediated toxicity to amacrine and bipolar cells in frog tadpoles increases the number of (3H)-thymidine incorporating cells at the CMZ for weeks after the injury (Reh, 1987). Neurotoxic injury to tyrosine hydroxylase-containing amacrine cells in tadpoles increases proliferation at the CMZ and directs cells to specifically replace the cell type destroyed by the lesion (Reh and Tully, 1986). In adult stages, amphibian retinal regeneration from the CMZ diminishes and occurs mainly by transdifferentiation of the pigmented epithelium. Still, some regeneration occurs at the peripheral margin of the retina even in adult stages (Mitashov, 1968; Keefe 1971, 1973; Reyer, 1971).

Early work in embryonic chicks demonstrated that retina can be regenerated from the peripheral margin of the eye after retinectomy, as long as a piece of neural retina is left behind in the eye cup (Coulombre and Coulombre, 1965). Further studies showed that a source of FGF-1 can be substituted for the piece of neural retina (Park and Hollenberg, 1991). Retinal regeneration from the CMZ in the embryonic avian eye proceeds quickly, and evidence of regeneration can be seen as early as one day after removal of the retina. Recent work has shown that FGF-2 and Sonic hedgehog are independently sufficient to induce regeneration from the CMZ after retinectomy (K. Del Rio-Tsonis, personal communication), while other known retinal mitogens such as TGF-β, insulin, IGF-1, and IGF-2 are each insufficient (Park and Hollenberg, 1991).

Unlike the CMZ of fish and amphibians, neurotoxic retinal damage does not affect BrdU incorporation of progenitors at the CMZ in posthatch chickens (Fischer and Reh, 2000). It is not obvious why the progenitors at the margin of the posthatch chicken retina respond differently to retinal injury. It is possible that retinal injury in the chicken does not result in the appropriate release of growth factors necessary for CMZ proliferation. The cells at the margin of the posthatch chicken are clearly capable of responding to growth factors (Fisher and Reh, 2000), and regeneration from the CMZ is possible in embryonic stages. The developmental reasons underlying the difference in regenerative potential between the CMZ in posthatch chicks and adult lower vertebrates is a subject for future study.

Although mammals have no CMZ, recent studies have shown that there is potential for neurogenesis at the retinal margin. As mentioned above, by studying mice with mutations in signaling pathways, we discovered that mice with a single allele of the Shh receptor, patched (ptc), have a small zone of proliferating cells at the retinal margin that is highly reminiscent of the CMZ of other vertebrates. When the ptc +/- animals were bred onto a retinal degeneration background (pro23his rhodopsin transgenic), there was a significant increase in the proliferation of CMZ cells at the retinal margin. Thus, the CMZ of the ptc +/- mice responded to retinal damage in a similar way to the CMZ of lower vertebrates. In addition, the BrdU-labeled cells from the margin were incorporated into the retina and expressed antigens of retinal neurons. Thus, while the normal mouse retina does not possess a distinct CMZ, partial activation of the Shh pathway enables such a zone to form, which, upon retinal damage, can show some signs of retinal cell regeneration (Moshiri and Reh, 2004).

Regeneration of retina from intrinsic cells

Cells within the retina proper can also be a source of regeneration in certain vertebrates. In addition to the progenitors located in the CMZ, the fish retina also has rod precursor cells in the outer nuclear layer (Johns and Fernald, 1981) and quiescent stem cells in the inner nuclear layer throughout the retinal circumference (Julian *et al.*, 1998). Experiments removing patches of central retina have shown that the border of the excised retina forms a blastema, which begins to proliferate and replace the removed retina. The source of these cells is not the CMZ, which is located far peripheral to the excision site, nor has transdifferentiation of pigmented epithelium been observed in fish (Raymond and Hitchcock, 1997). Rather, retinal regeneration in the central retina originates from the intrinsic rod progenitors of the ONL and the normally quiescent progenitors of the INL, both of which proliferate rapidly in response to the loss of cells around them (see Otteson and Hitchcock, 2003, for review). The discovery of the rod progenitor reconciled observations that the fish retina stretches during growth, yet maintains uniform rod density. Previous hypotheses of rapidly migrating rods from the retinal margin were discarded.

In addition to intrinsic stem cells and the cells of the retinal ciliary marginal zone, at least one non-neuronal retinal cell type, the Müller glia, exhibits progenitor-like behavior under certain circumstances. Retinal cell types are generated in a stereotyped order that is conserved among vertebrate species. Ganglion cells, cone photoreceptors, amacrine cells, and horizontal cells are born in the first wave, and bipolar neurons, rod photoreceptors, and Müller glia are generated in the second wave of differentiating cell types (Young *et al.*, 1985; Carter-Dawson and Lavail, 1979). Retroviral lineage-

tracing studies suggest that some late-stage retinal progenitors can differentiate as either rods or Müller glia, as some labeled two-cell clones contained both cell types in analyzed rat retinas (Turner and Cepko, 1987). Müller glial differentiation is dependent on some of the same molecular mechanisms that maintain progenitors in a multipotent state, namely Notch signaling.

The canonical Notch signaling pathway involves activation of the Notch receptor by a Delta-expressing cell. The intracellular domain of Notch is then cleaved, which allows it to translocate to the nucleus, interact with Supressor of Hairless and activate genes such as those in the Hairy/Enhancer of Split (Hes) family (see Baker, 2000, for review). Hes proteins, which are bHLH transcriptional repressors, can ensure that neurogenic bHLHs, among other genes, remain transcriptionally repressed, thereby inhibiting neuronal differentiation. The role that Notch signaling plays in maintaining the pluripotency of retinal progenitor cells has been demonstrated in both Xenopus and chicken (Dorsky *et al.*, 1997; Henrique *et al.*, 1997). But at least three groups have shown that

overexpression of Hes proteins in mouse retina results in more Müller glial production at the expense of neurons, and that a reduction in Hes levels or Notch signaling results in more neuronal differentiation at the expense of the Müller glia (Furakawa *et al.*, 2000; Hojo *et al.*, 2000; Satow *et al.*, 2001). These studies have led to speculation that Müller glia may be closely related to late-stage progenitors and therefore may retain the ability to dedifferentiate and become neurons.

Mature Müller glia span the retina from the vitreal surface to the retinal pigmented epithelium, exhibiting a bipolar shape and extending processes that interdigitate between retinal neurons. They express many of the proteins typical of astrocytes, such as glutamine synthetase (GS) and glutamate transporters, as well as voltage-gated channels and neurotransmitter receptors (see Newman and Reichenbach, 1996, for review). Under ordinary conditions, these cells remain quiescent and perform such glial functions as spatial ion buffering, structural support, and neurotransmitter uptake following neuronal excitation.
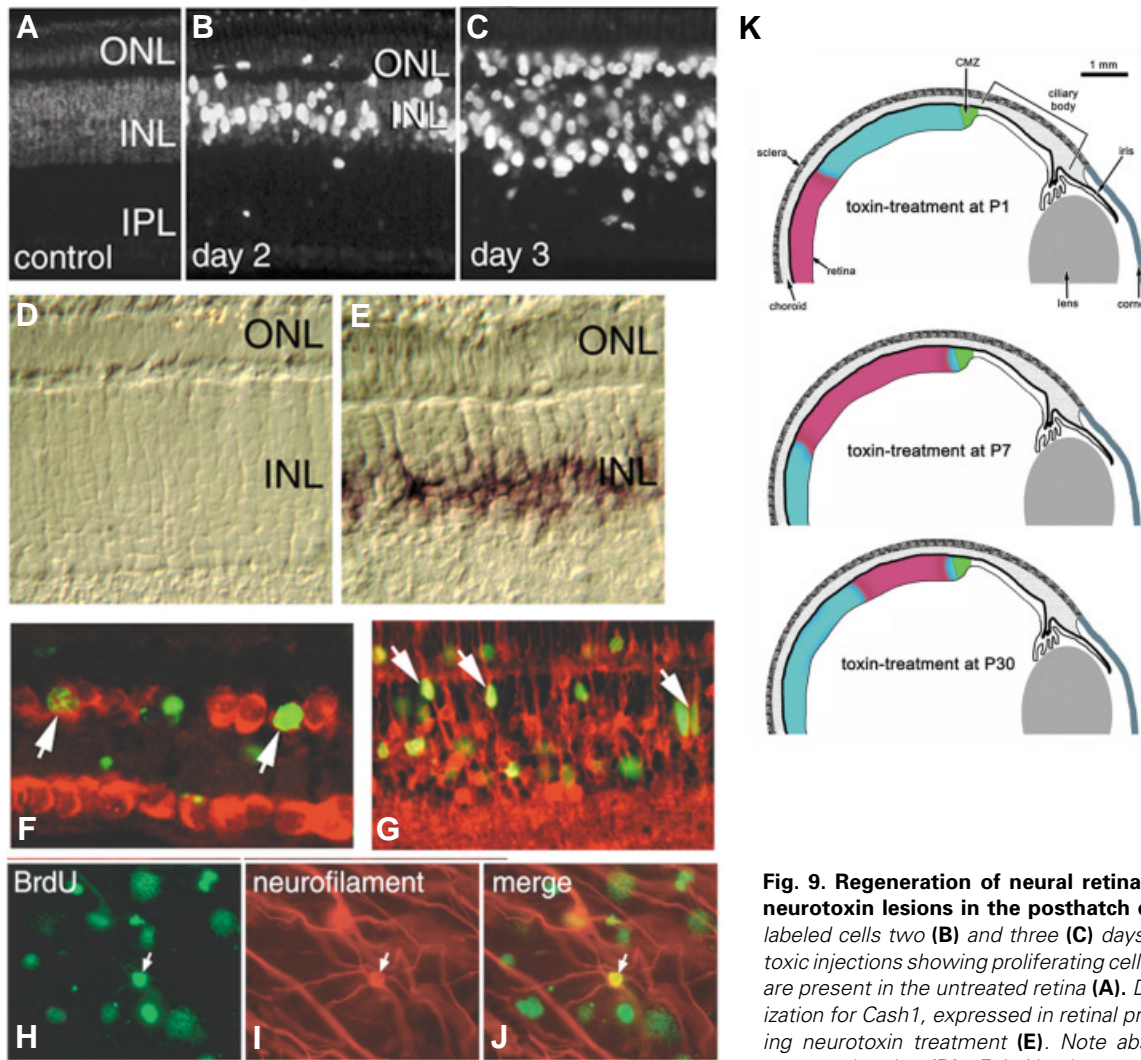
Fig. 9. Regeneration of neural retinal cells following neurotoxin lesions in the posthatch chick. *A-C. BrdU-labeled cells two* **(B)** *and three* **(C)** *days following neurotoxic injections showing proliferating cells. No labeled cells are present in the untreated retina* **(A)**. *D,E. In situ hybridization for Cash1, expressed in retinal progenitors, following neurotoxin treatment* **(E)**. *Note absence of label in untreated retina* **(D)**. *F-J. Newly generated neurons in posthatch chick retina following neurotoxin treatment, shown by double-labeling for BrdU (green) and the following neuron-specific markers (red):* **(F)** *Hu;* **(G)** *CRABP;* **(I,J)** *Neurofilament.* **(K)** *The region of regeneration (red) moves from central retina at P1 (top) to more peripheral regions at later stages (P7, middle, and P30, bottom panel). (A-G. from Fischer and Reh, 2001; H-J from Fischer* et al., *2002; K. from Fischer and Reh, 2003.)*

However, in fish and avian retina, and, to a lesser extent, in mammalian retina, Müller glia can behave in a decidedly progenitor-like fashion after damage or growth factor treatment. As mentioned previously, fish can regenerate retinal neurons throughout life, especially following damage, either from a marginal zone of stem cells or from a committed population of precursors in the central retina. In the adult goldfish retina, laser-induced retinal damage results in Müller glial migration to the outer nuclear layer, upregulation of glial fibrillary acidic protein (GFAP), and incorporation of BrdU (Braisted *et al.*, 1994; Wu *et al.*, 2001). This has led to some speculation that Müller glia might be capable of acting as neuronal precursors in the teleost retina (see Otteson and Hitchcock, 2003, for review). Müller glia of the avian retina also proliferate in response to damage. In the avian retina, N-methyl-D-aspartate (NMDA) injection normally produces massive cell death—particularly of amacrine cells—in addition to retinal thinning (Fischer *et al.*, 1998). In postnatal day 7 chicks, an age at which the retina is completely postmitotic, intraocular injection of NMDA, followed 48 hours later by a single injection of BrdU (Fig. 9A), reveals that more than half of the glutamine synthetase-positive Müller glia located in the inner nuclear layer enter S-phase after damage (Fischer and Reh, 2001). Furthermore, these BrdU-positive cells went on to exhibit a progenitor-like gene expression profile, with chicken achaete-scute homolog1 (Cash1) expression (Fig. 9) and Pax6/Chx10 co-expression, suggesting that these cells dedifferentiate following damage to the retina.

Fischer and Reh (2001) found that, while the majority of the Müller glia-derived, BrdU-positive cells remained undifferentiated after several days, about 20% became GS-expressing Müller glia, and a small percentage went on to express the neuronal markers Hu (Fig. 9C) and/or Cellular Retinoid Binding Protein (CRABP) (Fig. 9D), suggesting that they had acquired an amacrine or bipolar identity.

A similar Müller glial response was elicited by Fischer and colleagues after treatment with colchicine or kainic acid, using the same P7 chick injection experimental design, with a few notable exceptions (Fischer and Reh, 2002). Kainic acid treatment results in the death of amacrine, bipolar and ganglion cells, while colchicine induces ganglion cell death (Fischer *et al.*, 1999). After injection of these toxins, Müller glia entered the cell cycle in similar proportions to the NMDA experiments. But, unlike the NMDA-treated chick retinas, when retinas were treated with colchicine or kainic acid, some of the Müller glia-derived, BrdU-positive cells went on to express the ganglion cell markers Brn3 and/or Neurofilament (Fig. 9E). Taken together, these toxin injection experiments suggest that the Müller glia of the posthatch chicken (Fig. 9F) might retain the capacity to dedifferentiate and selectively replace neuronal types that are lost in the mature retina, thereby constituting a progenitor population.

Fischer and colleagues have also shown that Müller glia can act as progenitors in the absence of cell death when treated with a combination of FGF-2 and insulin (Fischer *et al.*, 2002). After three consecutive intraocular injections of 100 ng FGF-2 and 2 μg insulin, beginning at posthatch day 7, many BrdU-positive cells were observed in the margins of the retina. Furthermore, about 77% of the BrDU-positive cells that were located in the inner nuclear layer were also positive for the Müller glial marker GS within six hours after the final injection. However, within 24 hours, these cells had downregulated GS, suggesting dedifferentiation had taken place. Most of these growth factor-induced, BrdU-positive cells went on to form undiffer-

entiated Pax6-positive progenitor cells, but some went on to become Müller glia and a small percentage went on to express calretinin and/or Hu, two neuronal markers.

Müller glia possess surprising neurogenic capacity in the mature avian retina, but if mammalian retinal regeneration is to be achieved using Müller glia as a source of neurons, several obstacles must be overcome. First, very small numbers of these cells differentiated as retinal neurons after toxin treatment or growth factor injections. Future studies must find a means to boost neuron production from Müller glia in order to gain meaningful, functional regeneration. Second, photoreceptors have not been generated from Müller glia to date. The addition of intrinsic or extrinsic factors may be necessary to produce rods and cones. Finally, mammalian Müller glia have proven much more resistant to *in vivo* proliferation, with extremely small percentages of the population entering the cell cycle after toxin treatment or growth factor injections (Dyer and Cepko, 2000; Close, Gumuscu, and Reh, unpublished observations).

Conclusions

One of the most striking features of the research into retinal regeneration is the variety of cellular sources and mechanisms that can participate in the process. Even among the lower vertebrates, like amphibians and fish, the former regenerate the retina from the pigmented epithelium, while the latter have virtually no contribution from this source, and instead depend on the activation of a stem cell intrinsic to the retina. Perhaps the most unifying aspect of these diverse cellular sources is that they were all initially derived from the neural tube, and more specifically the optic vesicle, and it is likely that the neurogenic potential of the pigmented epithelium, ciliary epithelium and Müller glia is under active, and in some cases, reversible repression. The long term goal of encouraging regeneration in higher vertebrates to approach that of fish and amphibians may well lie in a better understanding of the molecular mechanisms underlying the repression of neurogenesis in the non-neuronal derivatives of the nervous system precursors.

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