Development/Plasticity/Repair

Persistent Progenitors at the Retinal Margin of *ptc*+/− Mice

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The hedgehog signaling pathway is a key regulator of neural development, affecting both proliferation and differentiation of neural progenitors. Sonic hedgehog (Shh) is a mitogenic factor for retinal progenitors *in vitro*. To determine whether this signaling system is important *in vivo* for regulating retinal progenitor proliferation, we analyzed mice with a single functional allele of the Shh receptor *patched* (*ptc*). We found that *ptc*+/− mice had increased numbers of neural progenitors at every stage of retinal development that we examined. In addition, these mice had persistent progenitors at the retinal margin for up to 3 months of age, reminiscent of the ciliary marginal zone of lower vertebrates. To test whether the progenitors at the retinal margin of *ptc*+/− mice could be induced to regenerate retinal neurons in response to damage, we bred *ptc*+/− mice onto a retinal degeneration background (pro23his rhodopsin transgenic) and labeled newly generated cells with combined immunohistochemistry for bromodeoxyuridine and retinal neuron and photoreceptor-specific markers. We found newly generated neurons and photoreceptors at the retinal margin in *ptc*+/−;pro23his mice. We propose that the Shh pathway may act as a regulator of both prenatal and postnatal retinal growth.

Key words: **development; regeneration; retina; neurogenesis; progenitor; stem cell**

Introduction

Sonic hedgehog (Shh) signaling is important in several aspects of neural development, affecting both proliferation and differentiation of cells throughout the CNS. Shh secreted by the notochord and the floor plate of the neural tube patterns its ventral differentiation (for review, see Ericson et al., 1995). Recent studies have established a mitogenic role for Shh signaling in CNS progenitor cells. Cerebellar granule cell precursors depend on Shh secreted by Purkinje cells to proliferate *in vitro* and *in vivo* (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Studies in granule cell precursors have shown that Shh, through direct transcriptional regulation of *N-myc*, upregulates *cyclin D1*, *cyclin D2*, and *cyclin E* mRNA, which promote entry into S phase (Kenney and Rowitch, 2000; Kenney et al., 2003). In addition to its mitogenic role in the cerebellum, Shh also promotes proliferation of embryonic neural progenitors in the spinal cord (Rowitch et al., 1999), midbrain (Britto et al., 2002; Ishibashi and McMahon 2002), forebrain (Dahmane et al., 2001; Britto et al., 2002), and retina (Jensen and Wallace, 1997; Levine et al., 1997; Wang et al., 2002).

Recently, Shh has also been implicated in adult neural stem cell proliferation (Lai et al., 2003). Adult neurogenesis in vertebrates has been well characterized in the subventricular zone of the cortex and the dentate gyrus of the hippocampus (Alvarez-Buylla et al., 2001). Lai et al. (2003) were able to modulate mitotic activity of these progenitors in adult rats *in vivo* by viral delivery of Shh or injection of cyclopamine, a potent Shh signaling inhibitor. Another well-documented neural stem cell zone exists at the retinal margin of nonmammalian vertebrates. The retinas of postembryonic frogs and fish grow significantly by the addition of new cells at the ciliary margin (Hollyfield, 1968; Straznicky and Gaze, 1971; Johns, 1977; Reh and Constantine-Paton, 1983), by a zone of stem cells known as the ciliary marginal zone (CMZ). Recent studies have shown that limited neurogenesis occurs at the retinal margin of posthatched birds as well (Fischer and Reh, 2000; Kubota et al., 2002). No such source of retinal neurons has been found in the mammal *in vivo* (Kubota et al., 2002); however, recent reports suggest that pigmented cells from the rodent ciliary body can transdifferentiate into cells with properties of neural stem cells (Tropepe et al., 2000).

To determine whether the Shh/ptc signaling system is important *in vivo* for regulating retinal progenitor proliferation and might be important in adult neurogenesis in the mammalian eye, we analyzed mice with a single functional allele of the Shh receptor *patched* (*ptc*). Secreted Shh binds to its membrane receptor, Patched, thereby relieving inhibition of its downstream signaling cascade. Because Patched inhibits Shh signaling in the absence of ligand, mutations in the *Patched* (*ptc*) gene activate the pathway constitutively, causing overgrowth of the neural tube (Goodrich et al., 1997). Homozygous ptc mutants die *in utero* at E9.5. Animals heterozygous for *ptc* have increased cerebellar proliferation and frequently develop medulloblastoma (Goodrich et al., 1997). In our analysis of the retinas of *ptc*+/− mice, we found that mice with a mutation in the *ptc* gene have an increased percentage of proliferating cells in their retinas throughout the first postnatal week. In addition, the mice have a population of dividing cells at the retinal margin reminiscent of the CMZ of lower vertebrates. The population of cells at the retinal margin of *ptc*+/− mice, like

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cells of the CMZ, proliferate in response to injury, express genes typical of the CMZ of lower vertebrates, and show a limited potential to regenerate retinal neurons. These results suggest that the hedgehog pathway may be a critical regulator of postembryonic ocular growth.

Materials and Methods

Analysis of mice. Mice were housed in the Department of Comparative Medicine at the University of Washington. All procedures were performed in accordance with approved protocols. The laboratory of Dr. M. P. Scott (Stanford University, Stanford, CA) created the *ptc*+/− mice, which were obtained from the Jackson Laboratory (Bar Harbor, ME). The pro23his rhodopsin transgenic animals were a gift from Dr. T. Dryja (Harvard University, Boston, MA). The analysis of the *ptc*+/− mice was done on litters that were killed at various ages. Each mouse was perfused with 2% paraformaldehyde, and the eyes were harvested. The lens was removed anteriorly from each eye, and the remaining ocular tissues, including the retina, were incubated in 2% paraformaldehyde for 1 hr. Subsequently, the retinas were incubated in 30% sucrose overnight at 4°C, frozen in optimal cutting temperature compound, cryosectioned at 14 μm, and analyzed with immunohistochemistry. Some of the mice received subcutaneous injections of 100 μl of 10 mg/ml bromodeoxyuridine (BrdU) daily on postnatal days (P) 13–15. Other animals received injections of 50 μl of 10 mg/ml BrdU every 2 hr for 48 hr during P14–15. Animals were killed 5 d after the last injection (P20) and prepared the same as above. Older animals received injections of 100 μl of 10 mg/ml BrdU daily on P85–89 and were killed on P90 and prepared as above. Animals were genotyped by PCR using genomic DNA from digestion of tail samples. The primers used for *ptc*+/− genotyping were f-GATGTTTCGCTTGGTGGTCGAATG and r-GCTGTTCTCCTCTTCCTCATCTCC. Primers used for genotyping of mice with the pro23his rhodopsin mutation were f-GAGTGCACCCTCCTTAGGCA and r-TCCTGACTGGAGGACCCTAC.

Immunohistochemistry. In this study, the following antibodies were used: (1) rat monoclonal anti-BrdU (1:200; Accurate Chemicals, Westbury, NY); (2) rabbit anti-phosphohistone H3 (PH3) (1:750; Upstate Biotechnology, Lake Placid, NY); (3) rabbit anti-recoverin (1:800; provided by Dr. J. Hurley, University of Washington, Seattle, WA); (4) mouse anti-TUJ-1 (1:1000; Zymed, San Francisco, CA); (5) mouse anti-opsin 4D2 (1:500; provided by Dr. R. Molday, University of British Columbia, Vancouver, British Columbia, Canada); (6) rabbit anti-chx10 (1:4000; provided by Dr. T. Jessell, Columbia University, New York, NY); (7) rabbit anti-nestin (1:1000; provided by Dr. R. McKay, National Institutes of Health); (8) rabbit anti-Brn3.2 (1:1000; provided by Dr. E. Turner, University of California–San Diego, La Jolla, CA); and (9) rabbit anti-Ki67 (1:2000; Vector Laboratories, Burlingame CA). The antibodies were used at the appropriate dilution in 0.2% Triton X-100 and 3% goat serum in PBS. Slides stained with anti-BrdU were incubated in 4N HCl for 15 min before incubation with the primary antibody. The primary antibodies were incubated overnight at 4°C on glass Superfrost slides (VWR Scientific) with cryosections that were encircled with a PAP pen (The Binding Site, San Diego CA). Species-specific secondary antibodies conjugated to fluorophores were used subsequently to visualize localization of the primary antibodies by fluorescent and confocal microscopy. Double-labeled sections were performed as described previously (Fischer and Reh, 2000). β-Galactosidase staining was done using standard methods. Images were taken using a SPOT digital camera.

Cell culture. P10 mice were killed with CO_2, and the eyes were dissected into sterile HBSS with HEPES buffer at 4°C. Neural retinas were dissected from the retinal pigment and other tissues. The anterior rim of the neural retina was dissected and dissociated by mild trituration after a 5 min incubation at 37°C in calcium–magnesium-free saline with trypsin (0.025%). Cells were plated onto coverslips in 24-well plates. Coverslips were coated sequentially with poly-D-lysine and Matrigel (1:100 dilution in HBSS; Collaborative Research, Bedford MA). Cultures were maintained at 37°C and 5% CO_2 for 5 d. The culture medium contained DMEM–F12 (without glutamate or aspartate), 25 μg/ml insulin, 100 μg/ml transferrin, 60 μM putrescine, 30 nM selenium, 20 nM progesterone, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.05 M HEPES, and 1% FBS (Invitrogen, San Diego, CA). One-half of the media in each well was changed every 48 hr. BrdU (10 μg/ml) and growth factors (20 μg/ml) were added on the first day of culture and maintained at the same concentration throughout the 5 d culture period. Epidermal growth factor (EGF) and basic FGFwere both obtained from R & D Systems (Minneapolis, MN). After 5 d in culture, coverslips were fixed in 4% paraformaldehyde for 1 hr and then rinsed in PBS before processing for immunohistochemistry as above. The number of labeled cells on each coverslip was quantified by counting all of the labeled cells in both a vertical and horizontal strip across the entire coverslip.

Cell cycle analysis. Retinas were dissected from animals and submerged in 1 ml of a buffer containing DAPI (10 μg/ml), NaCl (146 mM), Tris base (10 mM), CaCl2 (2 mM), MgCl2 (22 mM), BSA (0.1 mg/ml), NP-40 (0.1%), and DMSO (10%) in water. DAPI-labeled nuclei were passed through a flow cytometer that measured the peak intensity of the DAPI signal and the area of each particle passing through the sensor. Raw data were processed (WINCYCLE software) by gating to exclude clumps of nuclei and cell debris. The gated area contained >90% of the raw data points, indicating that nearly all of the dissociated tissue was preserved and analyzed. By measuring the amount of DAPI fluorescence, the amount of DNA in each nucleus was determined, and the proportion of cells in each segment of the cell cycle was quantified.

Quantitative PCR. RNA was obtained from neural retinas of P0 *ptc*+/− animals and wild-type littermates using TRIzol (Invitrogen) and cleaned using an RNeasy mini cleanup kit (Qiagen, Hilden, Germany). Superscript II Reverse Transcriptase (Invitrogen) was used to produce cDNA from each animal, and all samples were normalized with primers to glyceraldehyde-3-phosphate dehydrogenase. Quantitative PCR was performed in triplicate for each sample using SYBR Green PCR master mix (MJ Bioworks) with an Opticon monitor (MJ Research), and the cycle in which log phase was attained was recorded. Primers designed to the 3′ untranslated region of mouse Gli1 were f-CAGGTGTGTAACGCTCTGGA and r-TTGCTCATGGGAAAGAGGAG, and PCR product sizes were verified by gel electrophoresis.

Quantification of cell numbers. To determine the number of proliferating cells, litters of mice were sacrificed at several ages in the first postnatal week, and retinal sections from each mouse were labeled with anti-PH3 to identify cells in the G_2 and M phases of the cell cycle (Hendzel et al., 1997). To quantify the number of labeled cells, 14 μm sections were taken at the level of the optic nerve head. At P0, labeled cells were counted across a uniform length (310 μm) of central retina for each section. The average number of PH3-labeled cells per unit length of central retina was then calculated. Using the average total length of a retinal section, the total number of labeled cells was estimated per section. For older animals, all PH3-labeled cells per section were counted directly. The number of dividing cells at the margin of adult mice was determined by counting BrdU-labeled nuclei per retinal margin. The number of ganglion cells in P3 animals was determined by counting Brn3.2-positive nuclei in a 310 μm length of retinal sections of 14 μm thickness at the level of the optic nerve head. Total retinal thickness and outer nuclear layer (stained with rhodopsin) thickness of P3 animals was measured using 14 μm sections analyzed on NIH Image software. All sections were counted blind to the genotype of the mice. All error bars in the figures represent SE, and n represents number of animals. Data from *ptc*+/− and wild-type retinas were compared statistically using the appropriate Student's *t* test, in which $*p < 0.05$ and $**p < 0.005$ in each figure.

Results

ptc+/− mice have normal lamination and increased proliferation in the retina

Previous experiments have shown that Shh has several roles in retinal development in fish, chickens, and rodents, including retinal ganglion cell differentiation, rod photoreceptor differentiation, Muller glial development, and mitotic activity of the progenitor cells (for review, see Hartenstein and Reh, 2002). Targeted deletion of the Shh receptor *ptc* leads to an activation of the Shh signaling pathway (see Introduction). To directly verify that Shh signaling is increased in the retina, we sought to measure

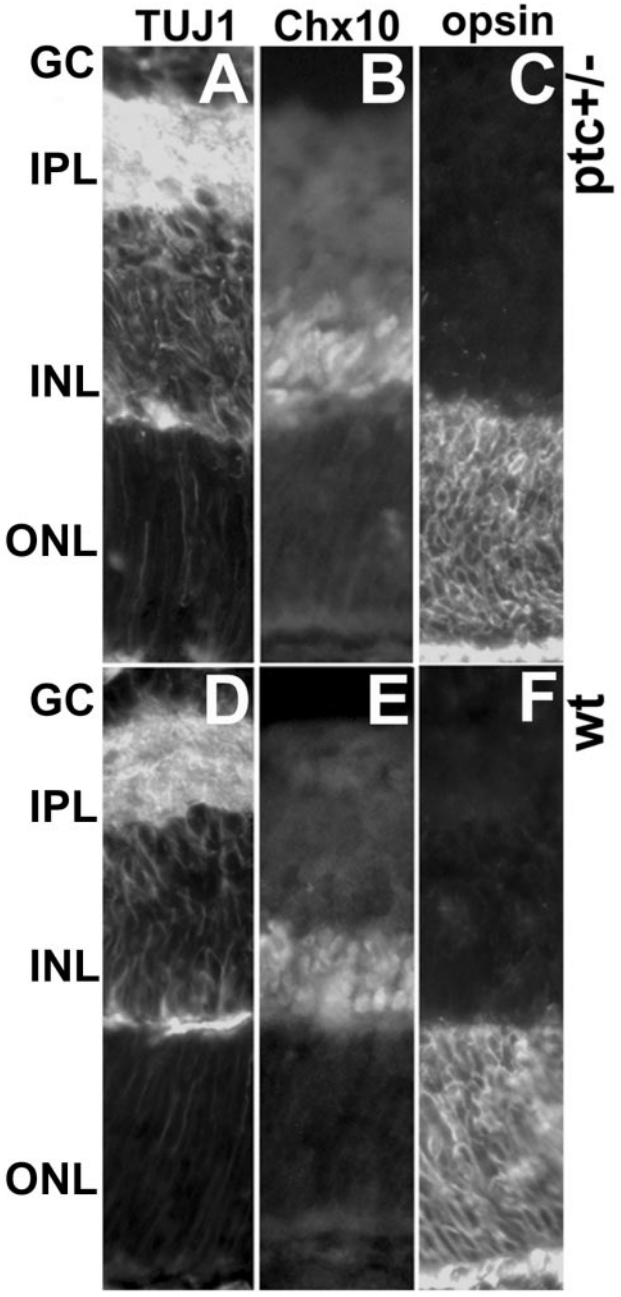

Figure 1. ptc+/− mice have a morphologically normal retina. *A–C*, P7 retinal sections from *ptc*+/− mice show typical retinal lamination indistinguishable from age-matched wild-type littermates (*D–F*). GC, Ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

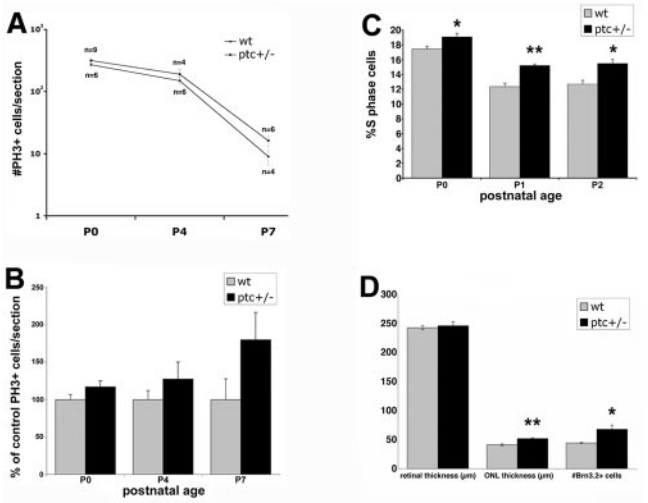

Figure 2. ptc+/− mice have increased retinal proliferation during development. *A*, The number of M phase PH3+ cells was consistently greater in the *ptc*+/− mice when compared with wild-type littermates. The average number of PH3+ cells per retinal section is plotted. *B*, The percentage of increase in PH3+ cells per section is shown, with wild-type normalized to one. The percentage of increase of M phase cells increases with postnatal age. *C*, Cell cycle analysis by flow cytometry reveals *ptc*+/− animals have an increased percentage of cells in S phase at each postnatal age studied. *D*, The total retinal thickness of P3 *ptc*+/− animals is the same as wild-type littermates, although the outer nuclear layer thickness (stained with rhodopsin) and the number of ganglion cells (stained with Brn3.2) at P3 are both significantly increased. $*p < 0.05$; $**p < 0.005$.

Gli1 transcript levels as a readout of Shh pathway activation. Gli1 expression is known to be regulated by Shh signaling (Marigo et al., 1996; Lee et al., 1997). Using quantitative PCR on cDNA from P0 neural retinas, we found a 75% increase ($p < 0.03$; Student's *t* test) in Gli1 mRNA levels in ptc+/− animals ($n = 3$) when compared with wild-type littermates ($n = 3$). To determine whether the partial activation of the Shh signaling pathway that occurs in *ptc*+/− mice was sufficient to disrupt any of these aspects of retinal development, we compared the retinas of *ptc*+/− mice with those of wild-type mice using immunohistochemistry for several different neuron-specific antigens during the first postnatal week. There were no clear differences in overall retinal development (Fig. 1). Markers for ganglion cells, inner retinal cell types (including amacrine cells and bipolar cells), and photoreceptors all showed their normal pattern and onset of expression. We also stained sections for GFAP, S100, and cellular retinaldehyde binding protein immunohistochemistry to determine whether the Muller glial cells were reactive in the ptc+/− retinas (data not shown) but found no difference from wild-type mice.

Although most of the retinal development appeared unperturbed in the *ptc*+/− mice, when we compared mitotic activity in the *ptc*+/− mouse retinas with that in retinas from wild-type animals, we found that there was an increase in several measures of cell proliferation. We compared the number of mitotic figures, using anti-PH3 antibodies to mark dividing cells in M phase, and found an increase in the number of labeled cells in the *ptc*+/− retinas (Fig. 2). To quantify this difference, we counted the number of labeled cells (see Materials and Methods) at three different postnatal ages. As shown in Figure 2*A*, *ptc*+/− mice had increased numbers of M phase cells at all ages we examined. Overall, in both wild-type and *ptc*+/− mice, the number of PH3+ cells declined with postnatal age because fewer cells remained in the cell cycle. The difference between the number of dividing cells in the two groups of mice increases with postnatal development. At birth (P0) the number of dividing cells is very similar between *ptc*+/− pups and their wild-type littermates. However, when older pups were examined, the difference in terms of percentage of control was greater (Fig. 2*B*).

To confirm the increase in retinal proliferation that we observed by immunohistochemical analysis, we used DNA content flow cytometry. Retinas from litters of *ptc*+/− mice were dissociated, labeled with DAPI, and passed through a flow cytometer (see Materials and Methods). After gating to exclude cell clumps, the proportion of cells in G_1, G_2, and S phase were quantified.

At each age we measured, there were consistently more cells in S phase in *ptc*+/− mice, indicating a higher number of replicat-

ing cells in their retinas when compared with wild-type littermates (Fig. 2*C*). As expected, the proportion of cells in the S phase of the cell cycle decreased with age both in wild-type and *ptc*+/− retinas. Nevertheless, *ptc*+/− pups had a 10–23% increase in S phase at each age. These data are consistent with those of the PH3 immunohistochemical analysis.

The increase in retinal progenitor proliferation in ptc+/− animals should cause an increase in the retinal size or the total number of cells in the retina. We measured the thickness of retinal sections taken at the level of the optic nerve head from P3 animals. We found that ptc+/− retinas were not significantly thicker than control. However, the thickness of the outer nuclear layer at this age was significantly greater in ptc+/− mice compared with wild-type littermates. We also quantified the number of ganglion cells in P3 animals. We found that P3 ptc+/− animals had significantly more Brn3.2-positive ganglion cell nuclei. Thus, although the overall size of the retina is not significantly larger in the ptc+/− animals, there are changes in the relative numbers of cells.

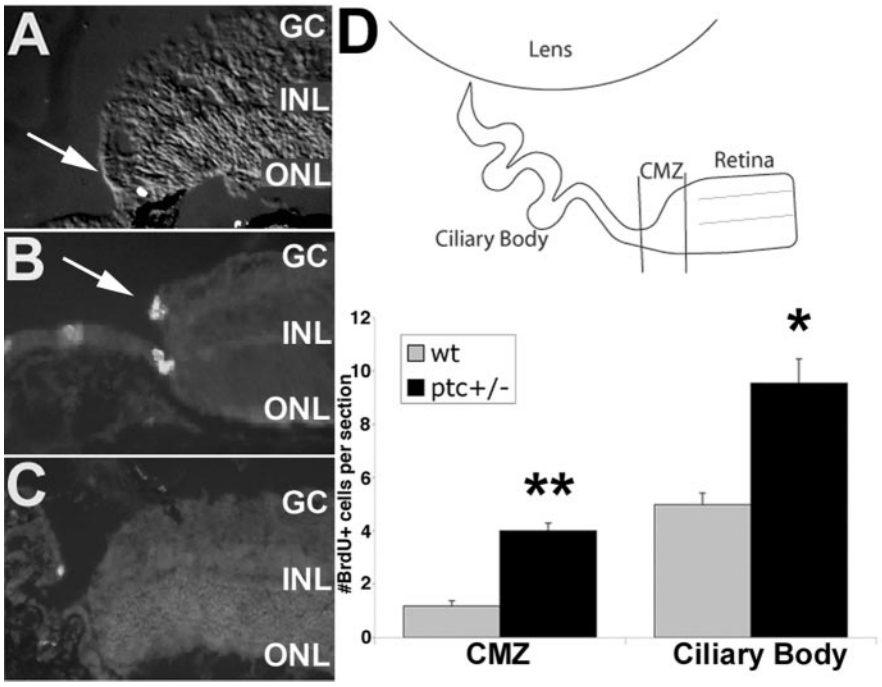

Figure 3. ptc+/− mice have extended proliferation at the retinal margin. *A*, P16 retina from *ptc*+/− mice has PH3-positive mitotically active cells (arrow). No PH3 labeling was observed in wild-type littermates. Sections of the peripheral margin of *ptc*+/− (*B*) and wild-type (*C*) mice at age P20, labeled for BrdU. The *ptc*+/− mice show proliferating cells at the retinal margin (arrow), whereas the wild-type mice have labeled cells nearly exclusively in the ciliary body. More frequent BrdU injections were made to analyze the number of dividing cells at the retinal margin. Animals received injections every 2 hr for 48 hr during P14–15 and were killed on P20. *ptc*+/− mice had more BrdU+ cells in the retina and in the nonpigmented epithelium of the ciliary body than wild-type littermates (*D*). Inset, Schematic diagram of the location of the BrdU+ cells that were counted. GC, Ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. $*p < 0.05$; $**p < 0.005$.

ptc+/− mice have a zone of cells at the retinal margin resembling a CMZ

Several previous studies have shown that retinal histogenesis is complete in the mouse by P11 in the periphery (Young, 1985). In our analysis of the P7 mice, we found that there were still some PH3+ cells in the periphery of wild-type retinas but many more in *ptc*+/− animals. To investigate how long neurogenesis continued in *ptc*+/− mice, we labeled retinal sections from animals up to 3 weeks after birth with PH3. We found that *ptc*+/− mice still had M phase cells near the retinal margin into their third postnatal week (Fig. 3*A*). To further assay whether the *ptc*+/− mice have a zone of persistent proliferation exclusively at the retinal margin, we made intraperitoneal injections of BrdU and subsequently processed the retinas for BrdU immunohistochemistry. Litters of pups received injections of BrdU on P13–15 and were killed 5 d later on P20. The *ptc*+/− mice had many BrdU-labeled cells at the retinal margin, whereas wild-type mice from these litters exhibited only an occasional BrdU-labeled cell (Fig. 3*B*,*C*). In addition to the difference in the number of BrdU-labeled cells between the *ptc*+/− and wild-type mice, we also found a difference in the position of the cells. The BrdU-labeled cells in wild-type mice were almost exclusively in the ciliary body with only the rare BrdU-positive cell in the retina proper. In the *ptc*+/− mice, BrdU labeling was consistently in the extreme periphery of the neural retina in every section in addition to the ciliary body (Fig. 3*B*,*C*). We did not observe BrdU labeling in the central retina in any animals.

To determine whether the daily injections of BrdU were sufficient to label all the dividing cells, we increased the frequency of BrdU injection. Mice received injections for 48 hr during P14–15 every 2 hr to maximize the number of labeled cells. Even in the mice that received injections of BrdU more frequently, we found many more BrdU-labeled cells in the *ptc*+/− mice than in wild-type littermates, both in the peripheral retina and ciliary body (Fig. 3*D*). The persistence of dividing cells at the retinal margin in the *ptc*+/− mice is reminiscent of the CMZ of other vertebrates.

To further characterize the proliferating cells at the retinal margin in the *ptc*+/− mice, we labeled retinal sections for several different markers of retinal progenitors including *ptc*, Chx10, and nestin. The mice that we used in this study had a β-galactosidase gene in place of a normal *ptc* allele. Thus, lacZ staining showed the distribution of *ptc* gene expression. Retinal progenitors in the neuroblast layer of newborn mice express *ptc* (Fig. 4*A*). *Ptc*+/− mice have a lacZ label in a small group of cells at the junction between the ciliary body and the retina (Fig. 4*B*), consistent with these cells being retinal progenitors. Chx10 is an antigen expressed in retinal progenitor cells in developing retina and retinal bipolar cells in the mature retina. In the normal mouse at P20, Chx10 is expressed in bipolar cells (inner nuclear layer) but not in the cells of the ciliary body (Fig. 4*C*). However, in the ptc+/− mice, a zone of cells coincident with the BrdU-incorporating nuclei is labeled with Chx10 antibody at the retinal margin extending into the ciliary body epithelium (Fig. 4*D*). Chx10 staining has been observed in a similar region in the chick eye, and it may correlate with the fact that this region of the ciliary epithelium is capable of generating neurons (Fischer and Reh, 2003). We also used antibodies against the neural progenitor marker nestin. *ptc*+/− retinas have a bright region of nestin staining precisely at the boundary of the retina and ciliary body, whereas wild-type littermates express nestin at a much lower level in a few scattered cells. Both *ptc*+/− and wild-type retinas also show a low level of nestin immunoreactivity in what we presume to be Muller glia near the retinal margin, as is evident from the sparse

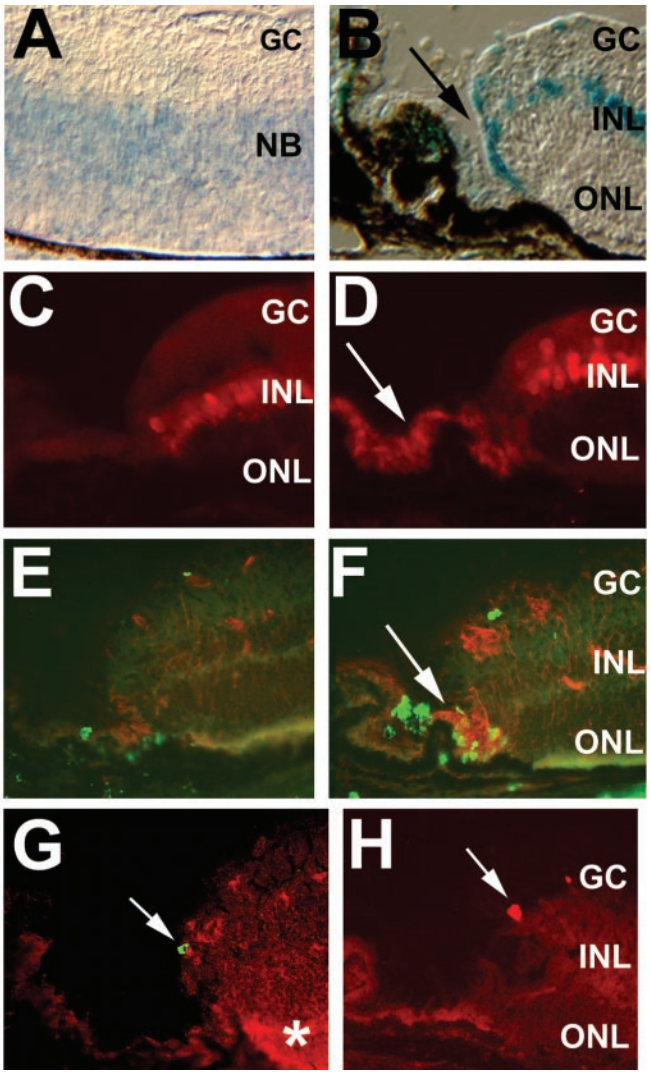

Figure 4. Proliferating cells at the retinal margin of *ptc*+/− mice are retinal progenitors. *A*, *ptc* is a retinal progenitor marker expressed in nearly all progenitors in the neuroblast layer of P0 retina. Proliferating cells at the retinal margin (*B*, *D*, arrows) of P20 *ptc*+/− mice express the progenitor markers *ptc* (*B*) and Chx10 (*D*), whereas wild-type littermates do not (*C*). Wild-type mice have very few BrdU-incorporating cells at the margin of the retina. Sections of the peripheral margin of wild-type (*E*) and *ptc*+/− (*F*) mice at age P20, labeled for BrdU (green), show a population of dividing cells at the *ptc*+/− retinal margin (*F*). The same sections stained with antibodies against the neural progenitor marker nestin (red) show a distinct population of labeled cells at the *ptc*+/− (*F*) retinal margin, but not in wild-type (*E*) littermates. These nestin-positive cells in *ptc*+/− mice are coincident with BrdU incorporation, identifying the proliferating cells at the *ptc*+/− retinal margin as undifferentiated progenitors (F, arrow). ptc+/− mice continued to incorporate (*G*) BrdU (green) and (*H*) Ki67 (red) at the retinal margin even at P90 after daily injections of BrdU on P85–89. These BrdU-positive cells also express nestin (arrow in G), and other nestin (red)-expressing progenitors are present at the retinal margin of these mice. No BrdU, nestin, or Ki67 was detected in the retinas of wild-type P90 littermates (data not shown). The asterisk indicates staining of an artifactual space between the outer nuclear layer and retinal pigment epithelium as a result of sectioning. GC, Ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

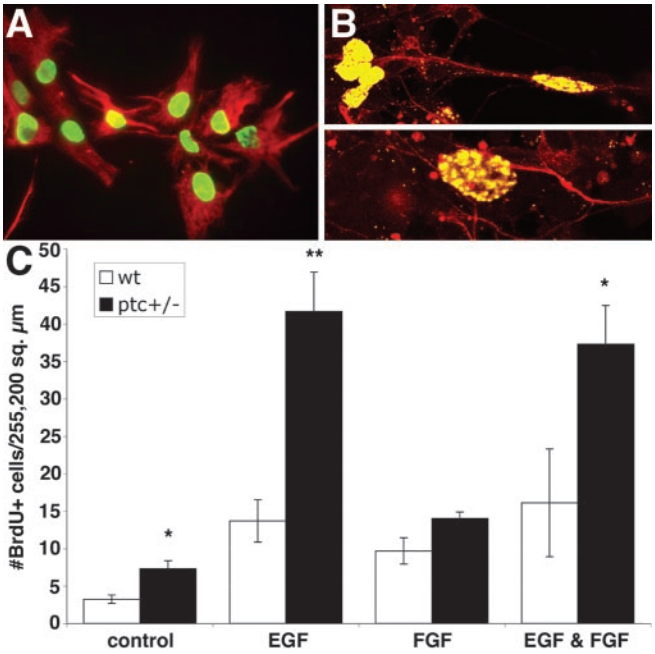

Figure 5. Progenitors from the retinal margin of *ptc*+/− mice proliferate in response to growth factors and differentiate into neurons. Anterior neural retinas from P10 *ptc*+/− mice and wild-type littermates were grown in dissociated cultures for 5 d. *A*, Ninety-five percent of BrdU-positive (green) cells in these cultures express the progenitor marker nestin (red). *B*, Other BrdU-positive cells had neuronal morphology and express neuronal genes, such as Tuj-1 (red), as confirmed by confocal microscopy. *C*, Similar to CMZ-derived progenitors in other species, progenitors from P10 *ptc*+/− mice proliferated in response to growth factors such as EGF and basic FGF during the 5 d culture period. $*p < 0.05$; $**p < 0.005$.

filamentous staining. Other work from our laboratory has shown that immature Muller glia have some potential for neurogenesis in birds (Fischer and Reh, 2001), and the nestin expression may reflect this. These nestin-expressing cells at the margin colocalize with the cells that incorporate BrdU (Fig. 4*E*,*F*). To assess whether or not progenitors at the retinal margin of *ptc*+/− mice continue to divide late in adulthood, mice received injections of BrdU daily during P85–89 and were killed on P90. We found that BrdU incorporation persisted, although at a significantly lower level, at the retinal margin of *ptc*+/− mice. The BrdU-labeled cells also expressed nestin (Fig. 4*G*). In addition, cells in this location expressed the proliferation marker Ki67 (Fig. 4H). No BrdU-, nestin-, or Ki67-labeled cells were found in wild-type P90 animals at the retinal margin. These data further support the hypothesis that the loss of one allele of the *ptc* gene has allowed a region of retina in mice to remain in a state resembling the CMZ of lower vertebrates, both in its proliferative potential and in its gene expression.

Adult retinal progenitors at the CMZ are known to proliferate in response to growth factors (Reh, 1989). For example, injection of EGF, insulin, and IGF-I into posthatched chicken eyes induce proliferation of CMZ progenitors (Fischer and Reh, 2000). To determine whether progenitors at the margin of *ptc*+/− retinas respond to growth factors in a similar way, we cultured dissociated retinas from P10 animals, an age in which nearly all retinal cells in wild-type mice are postmitotic (Young, 1985). As expected, the vast majority of the dissociated retinal cells did not survive in culture, but some did proliferate and survive in each condition. We found that cultures from *ptc*+/− mice proliferated more than wild-type littermates as assayed by BrdU incorporation. In addition, the progenitors from *ptc*+/− retinas were stimulated to proliferate in response to FGF and EGF, similar to CMZ-derived retinal progenitors in other species (Fig. 5*C*). Over 95% of the BrdU+ cells in these cultures expressed the progenitor marker nestin (Fig. 5*A*). Some BrdU+ cells from the *ptc*+/− cultures expressed Tuj-1 after 5 d, as confirmed by confocal microscopy, indicating their potential to produce retinal neurons (Fig. 5*B*).

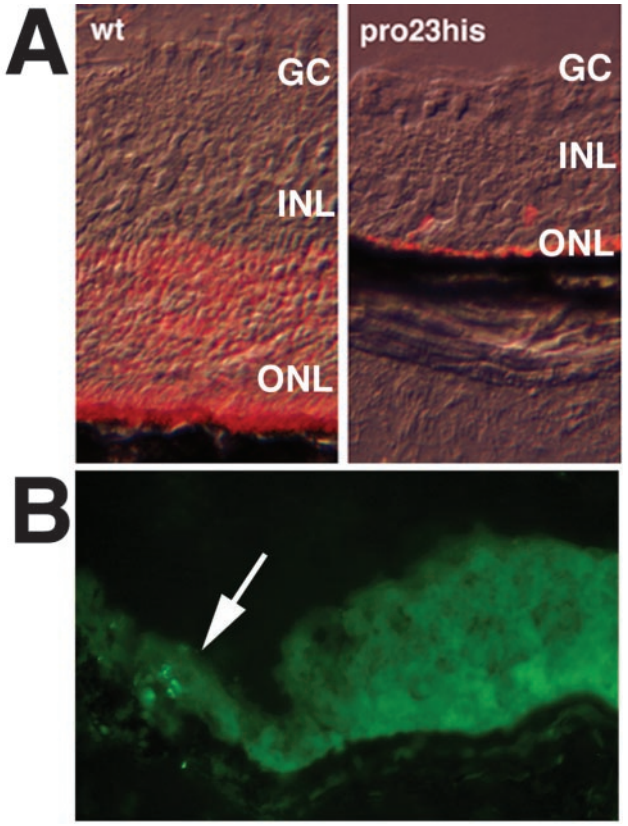

Figure 6. pro23his mice undergo severe photoreceptor degeneration. pro23his mice transgenically overexpress a mutated rhodopsin gene that causes severe photoreceptor death (Olsson et al., 1992). *A*, Recoverin (red), a photoreceptor marker, labels the outer nuclear layer (ONL). pro23his animals generate an ONL, which degenerates to a monolayer by P20. *B*, P20 pro23his animals that received injections of BrdU daily from P13–15 did not have increased proliferation at the retinal margin compared with wild-type littermates. Arrow indicates BrdU (green)-positive cells in the ciliary body epithelium. GC, Ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

ptc+/− mice have a limited capacity for retinal regeneration

Previous work in fish and frogs has shown that retinal injury causes an upregulation of proliferation in the CMZ and consequent retinal regeneration (reviewed by Reh and Levine, 1998; Reh and Fischer, 2001). We, therefore, asked whether damage or degeneration of retinal cells would have a similar effect on the cells of the retinal margin in the *ptc*+/− mice. To test for this possibility, we bred the *ptc*+/− mice onto a pro23his retinal degeneration background. The pro23his mice express a rhodopsin transgene with a single amino acid mutation at the 23rd position, replacing a proline with a histidine. The pro23his mice exhibit rapid and nearly complete degeneration of retinal photoreceptors by P20 (Olsson et al., 1992; Streichert et al., 1999). The mice develop a morphologically normal outer nuclear layer, which rapidly undergoes cell death, leaving only a monolayer of cone photoreceptors (Fig. 6*A*).

Litters bred from the two strains received intraperitoneal injections of BrdU on P13–15, after the completion of normal retinal histogenesis and were killed 5 d later on P20. Retinas of the animals from the genetically crossed litters were examined for any changes in the number of BrdU-labeled cells. Pro23his animals, like wild-type littermates, had very few BrdU-incorporating cells at the retinal margin (Figs. 6*B*, 8). *Ptc*+/− mice on the pro23his background exhibited the typical degeneration of the outer nuclear layer seen in pro23his animals (Fig. 6).

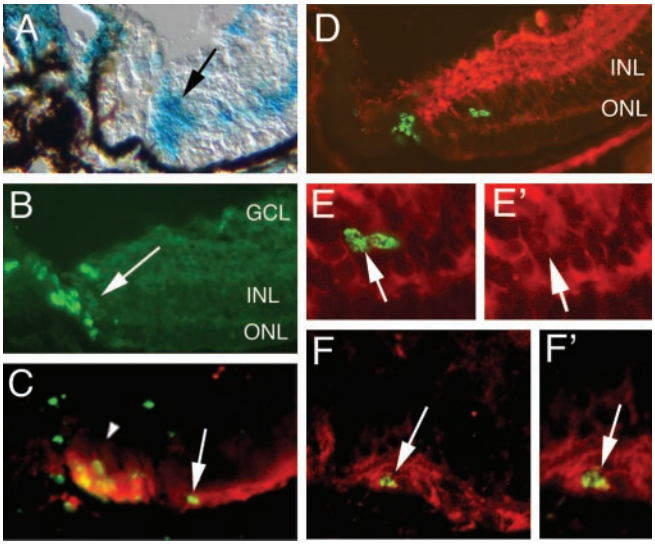

Figure 7. ptc+/− progenitors proliferate more, upregulate progenitor markers, and regenerate neurons in response to injury. *ptc*+/− and pro23his mice were mated to produce animals with both genotypes. *ptc*+/−;pro23his mice (P20) continued to express the progenitor markers at the retinal margin. *A*, *ptc* expression is markedly increased (arrow) at the retinal margin. Mice received injections of BrdU on P13–15. At P20, tissue was analyzed for the presence of BrdU-labeled proliferating cells at the retinal margin and with additional immunohistochemical markers to identify newly generated photoreceptors. Tissue was stained with markers for BrdU (green) and recoverin (red) to label photoreceptors. Progenitors at the margin (arrow) of *ptc*+/−;pro23his mice (*B*) proliferate more than in *ptc*+/− alone. Examples (*C*) are shown of double-labeled, newly generated photoreceptors (arrows). Double-labeled cells (arrow) were confirmed by confocal microscopy (*F*, *F'*). BrdU (green)-positive progenitors are capable of differentiating into other retinal neurons in addition to photoreceptors, including Tuj-1 (red)-expressing cells (*D*, *E*, *E'*). GC, Ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

However, the *ptc*+/−;pro23his mice show increased numbers of dividing cells at the retinal margin compared with animals of the *ptc*+/− genotype. Figure 7, *B* and *C*, shows an example of the retinal margin from the mice, whereas Figure 8 shows a graph of

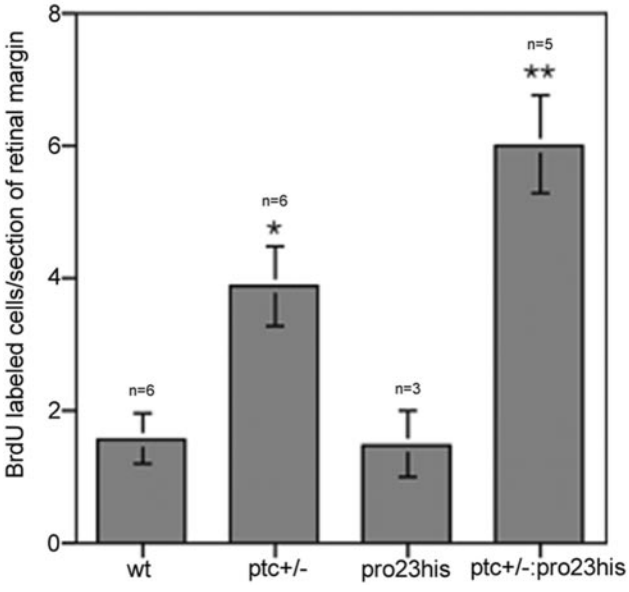

Figure 8. Cells in the *ptc*+/− retinal margin proliferate more in response to injury. The number of BrdU-labeled cells at the retinal margin was quantified in each of four mouse genotypes at age P20. Concomitant photoreceptor death stimulates progenitors at the margin of *ptc*+/− mice to divide. *$p < 0.05$; **$p < 0.005$.

the numbers of BrdU-labeled cells comparing the two genotypes. The retinal margin of the *ptc*+/−;pro23his mice has over 50% more BrdU-labeled cells than that of the *ptc*+/− animals.

As noted above, we were able to visualize the cells expressing *ptc*+/− by lacZ histochemistry. Although the *ptc*+/− mice show a few cells at the retinal margin that express the lacZ, the number of these cells is greatly increased in the *ptc*+/−;pro23his mice (Fig. 7*A*). In addition, the *ptc*+/−;pro23his mice have a large region of cells that continue to express the transcription factor Chx10 at the retinal margin (data not shown). The cells of the CMZ provide one of the sources of retinal regeneration in lower vertebrates (Reh and Levine, 1998; Reh and Fischer, 2001). To determine whether the proliferating cells at the retinal margin of the *ptc*+/−;pro23his mice may be differentiating to replace some of the dying photoreceptors, we labeled retinal sections with antibodies against BrdU and other markers of retinal neurons. Because the pro23his rhodopsin mutation causes degeneration of rod photoreceptors in these transgenic animals, using a rhodopsin antibody to label newly generated rods is problematic. Instead, we analyzed expression of recoverin, which is only expressed in rods, cones, and a small number of cone bipolar cells. In addition, most of the other photoreceptor markers are expressed late in their development, and the cells may degenerate before expressing these markers. Double-label experiments using BrdU and recoverin revealed that, indeed, some BrdU-labeled cells also express photoreceptor markers (Fig. 7*C*,*F*,*F*'). Colabeling of single cells was confirmed by confocal microscopy (Fig. 7*F*,*F*'). These cells were typically found in the outer nuclear layer very close to the retinal margin, but occasionally they appeared to have migrated tens of micrometers into the retina. Additional experiments showed that the progenitors at the margin of the retina differentiate into other retinal neurons in addition to photoreceptors. We detected BrdU in the nuclei of some Tuj-1-labeled cells in the inner retina, indicating the capability of progenitors at the retinal margin to differentiate into ganglion cells or amacrine cells (Fig. 7*D*,*E*,*E*'). These results indicate that the proliferating cells at the retinal margin of *ptc*+/− mice are likely persistent retinal progenitors.

Discussion

Previous studies have implicated hedgehog signaling in retinal progenitor proliferation (Jensen and Wallace, 1997; Levine et al., 1997; Wang et al., 2002; Perron et al., 2003) In this study, we have found that *ptc*+/− mice show two abnormalities in cell proliferation. First, the *ptc*+/− mice have a significant increase in the number of proliferating cells at postnatal ages when predominantly late-born retinal cell types are differentiating. These include the rod photoreceptors, the bipolar cells, and the Muller glia. Second, and more strikingly, we found that *ptc*+/− mice have a zone of persistent proliferation at the retinal margin reminiscent of the CMZ of fish and amphibians. These cells expressed markers of retinal progenitors including Chx10, *ptc*, and nestin. In addition, these cells responded to photoreceptor degeneration by increasing their proliferation, similar to cells in the CMZ of frogs and fish. Finally, we found that some of the progeny of the proliferating cells differentiate into photoreceptors, as indicated by their expression of the photoreceptor-specific protein recoverin and their laminar position, whereas other cells differentiate into Tuj-1-expressing cells in the inner retina.

Shh is an important mitogen in the CNS

Our finding that *ptc*+/− mice have an increase in retinal progenitor proliferation is consistent with findings in other areas of the CNS. Hedgehog signaling has been shown previously to drive cell proliferation in several regions of the developing nervous system (Goodrich et al., 1997; Rowitch et al., 1999; Dahmane et al., 2001; Ishibashi and McMahon, 2002). In the spinal cord, Shh has been overexpressed in embryonic mice using GAL4/upstream activating sequence methodology to study the effects of extended expression of Shh *in vivo*. These studies showed that Shh caused overproliferation of spinal cord precursors while keeping them in an undifferentiated state (Rowitch et al., 1999). The *in vivo* results in the spinal cord agree with previous experiments showing a proliferative response to Shh in spinal cord precursors in primary cell culture conditions (Kalyani et al., 1998). In the cerebellum, Purkinje cells express Shh, and developing granule neurons express *Ptc* (Wallace, 1999; Wechsler-Reya and Scott, 1999). Blocking Shh signaling with neutralizing antibodies *in vitro* and *in vivo* inhibits the production of granule cells, whereas the addition of Shh to granule cell cultures *in vitro*, or injected *in vivo*, stimulates granule cell genesis (Dahmane et al., 1999). Shh signaling is also important in proliferation of neural cells of the cortex and tectum. Reverse transcription-PCR experiments have located *Shh* mRNA in the cortex and tectum of E-13.5 mice, with continued expression through P5. *Shh* is expressed in progenitors of the subventricular zone (Charytoniuk et al., 2002) and stimulates proliferation of precursor cells in the ventricular zones of the telencephalon and mesencephalon and normal levels of proliferation can be attenuated with Shh inhibitors (Dahmane et al., 2001). More recent experiments have directly linked Shh signaling to proliferation of adult neural stem cells in the rat hippocampus. Hippocampal progenitors express *ptc*, and their proliferation can be increased more than threefold via adeno-associated virus-mediated Shh gene transfer to these cells. Normal adult hippocampal neurogenesis is inhibited by cyclopamine administration, which potently inhibits Shh signaling (Lai et al., 2003).

Mechanism of ptc regulation of cell proliferation

The molecular mechanisms of cell cycle regulation by Shh are not fully understood. Nevertheless, some facts are known about the role of Shh in cell cycle control. In granule cell precursors, the effects of Shh on proliferation seem to act through the cyclin–Rb axis. Cultures grown in the presence of Shh have increased expression of *cyclin D1*, *cyclin D2*, and *cyclin E*. In addition, the proliferative response to Shh is independent of the MAP kinase pathway, because the cells were unresponsive to MAP kinase kinase inhibitors (Kenney and Rowitch, 2000). Transcriptional regulation of cyclins by Shh is indirect, and subsequent studies have shown that Shh directly turns on the immediate early gene *N-myc*, which then directly induces cyclin expression (Kenney et al., 2003). These effects on cell cycle regulators may also be direct, because two-hybrid studies have shown that Ptc associates with phosphorylated cyclin B1 (Barnes et al., 2001). These authors proposed that Ptc binds phosphorylated cyclin B1 to prevent cell cycle progression. When Shh binds Ptc, cyclin B1 is released, allowing it to enter the nucleus and allowing the cell cycle to progress. Interestingly, in skin cell cultures, Shh is also able to override the inhibitory effect of cyclin-dependent kinase inhibitors such as p21(CIP1/WAF1) (Fan and Khavari, 1999). Although we have not yet examined whether similar mechanisms might be responsible for the observed effects on retinal progenitor proliferation in *ptc*+/− mice, several studies have previously shown a role for cyclin D1 in retinal histogenesis (Fantl et al., 1995; Sicinski et al., 1995).

Hedgehog in retinal progenitor proliferation

Our results confirm previous *in vitro* and *in vivo* studies documenting a role for hedgehog in retinal progenitor proliferation (Jensen and Wallace 1997; Levine et al., 1997). Exogenously applied Shh in cell culture stimulates proliferation of retinal progenitors of rats and mice, as evidenced by increased BrdU uptake. This effect is especially evident in photoreceptors and Muller glia, and it has been shown that Shh signaling promotes rod cell fate. We also found that the *ptc*+/− mice had a thicker outer nuclear layer and a greater number of ganglion cells. These results are consistent with the evidence from *in vitro* studies showing that Shh promotes rod photoreceptor development and ganglion cell formation. Rodents express sonic and desert hedgehog in the neural retina and Indian hedgehog in the retinal pigmented epithelium (Jensen and Wallace 1997; Levine et al., 1997). Recently, tissue-specific knock-out animals have shed more light on the role of Shh in the retina. Mice lacking Shh in retinal ganglion cells have smaller retinas than littermates, consistent with the role of Shh in retinal progenitor proliferation (Wang et al., 2002). As noted above, *ptc* is expressed in proliferating neuroblasts. The pattern of hedgehog regulation of histogenesis in the retina and cerebellum is strikingly similar. In both cases, the early neurons, retinal ganglion cells and cerebellar Purkinje cells, produce hedgehog to stimulate progenitors to proliferate (Wallace, 1999).

The role of ptc in the CMZ

The most striking finding of this study is that *ptc*+/− mice have a persistent zone of immature, mitotically active cells at the retinal margin reminiscent of the CMZ of lower vertebrates. We found that BrdU injections in mice up to 90 d after birth labeled cells in this zone. These cells also expressed markers of progenitors, including *ptc*, Chx10, and nestin. The progenitors at the retinal margin of chickens proliferate in response to growth factors (Fischer and Reh, 2000). To determine whether the putative progenitors at the margin of *ptc*+/− mice respond similarly to growth factor treatment, we cultured dissociated retinal cells from P10 mice under various conditions. We found that *ptc*+/− retinal cultures had increased BrdU uptake compared with littermate controls. In addition, 95% of the BrdU-positive cells in these cultures expressed nestin, a neural progenitor marker. Double-label experiments analyzed by confocal microscopy confirmed these BrdU-incorporating cells can differentiate into Tuj-1-expressing neurons.

The cells of the CMZ in lower vertebrates are responsible for normal retinal growth as the animal grows as well as for regeneration of retinal cells in response to injury (Reh and Nagy, 1987). To test whether the CMZ cells of *ptc*+/− mice were capable of regenerating retinal neurons, we bred them onto the pro23his photoreceptor degeneration background. We found that the cells at the retinal margin responded similarly to experiments done in nonmammalian species. In response to photoreceptor degeneration, the immature cells at the margin increased in proliferation by ~50%, and some differentiated into cells that express the neuronal marker Tuj-1 or the photoreceptor protein recoverin.

It has been known for many years that fish and frogs increase the size of their eyes during normal development. The retina in these organisms has been shown to grow as well, alongside the ocular growth. Stem cells at the retinal margin of fish and frogs provide a source of all retinal cell types, which incorporate seamlessly as the eye grows (for review, see Reh and Fischer, 2001). Lineage studies suggest that a slowly dividing more primitive stem cell is located far peripherally and capable of producing all retinal cell types as well as pigmented epithelial cells, and that faster cycling, more fate-restricted progenitor cells are located immediately adjacent to the retinal margin (Wetts et al., 1989). More recent work in the frog eye has directly shown expression of Shh signaling molecules in the CMZ, strongly implicating this signaling system in adult retinal proliferation in amphibians (Perron et al., 2003).

Recently, the margin of the posthatched avian retina has been reported to have cells that incorporate BrdU (Fischer and Reh, 2000; Kubota et al., 2002). These cells express transcription factors such as Pax6 and Chx10 that are common in retinal progenitors. Double-labeling studies with BrdU and neuron-specific markers have shown that the cells at the retinal margin of the chick can also generate retinal neurons. However, the CMZ of the bird is substantially smaller than that of the fish or frog and generates far less retinal tissue. Thus, it seems that the potential of the CMZ has been progressively reduced in higher vertebrate evolution, with some capability persisting in birds, but no such proliferative zone in normal rodents.

Recent reports indicate that the rodent eye contains retinal stem cells capable of proliferating and expressing markers of retinal neurons in cell culture (Ahmad et al., 2000; Tropepe et al., 2000). Both groups found that dissociated pigmented cells of the ciliary body of adult rodents proliferated in the presence of growth factors to form colonies expressing nestin and Chx10. After 3 weeks in culture, these cells expressed some markers of retinal neurons and glia. Neither pigmented cells from the posterior eye nor nonpigmented cells from the ciliary body had this potential. Neither group found dividing cells in the retina proper as described in *ptc*+/− mice. Taken together with our results, it seems that normal mice do not have retinal progenitors in the nonpigmented ciliary epithelium like frogs and fish; however, the *ptc*+/− mice retain these cells for some time.

The presence of what appears to be a retinal CMZ capable of producing neurons after injury in a mammal is very intriguing. This observation is especially noteworthy because it appears after the loss of one allele of *ptc*, creating a partial activation of the Shh signaling pathway. This suggests that decreased Shh signaling in the retina may be involved in the evolutionary restriction and eventual extinction of progenitors at the margin of the mammalian retina. It may also be that a complex set of genetic variables controls whether or not cells at the retinal margin are capable of dividing in adulthood as in amphibians and other lower vertebrates. Here, we show that increased Shh signaling is able to tip the balance in favor of producing a progenitor zone in the rodent retina reminiscent of the CMZ.

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