The hedgehog pathway is a modulator of retina regeneration


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Summary

The embryonic chick has the ability to regenerate its retina after it has been completely removed. Here, we provide a detailed characterization of retina regeneration in the embryonic chick at the cellular level. Retina regeneration can occur in two distinct manners. The first is via transdifferentiation, which is induced by members of the Fibroblast growth factor (Fgf) family. The second type of retinal regeneration occurs from the anterior margin of the eye, near the ciliary body (CB) and ciliary marginal zone (CMZ). We show that regeneration from the CB/CMZ is the result of proliferating stem/progenitor cells. This type of regeneration is also stimulated by Fgf2, but we show that it can be activated by Sonic hedgehog (Shh) overexpression when no ectopic Fgf2 is present. Shh-stimulated activation of CB/CMZ regeneration is inhibited by the Fgf receptor (Fgfr) antagonist, PD173074. This indicates that Shh-induced regeneration acts through the Fgf signaling pathway. In addition, we show that the hedgehog (Hh) pathway plays a role in maintenance of the retina pigmented epithelium (RPE), as ectopic Shh expression inhibits transdifferentiation and Hh inhibition increases the transdifferentiation domain. Ectopic Shh expression in the regenerating retina also results in a decrease in the number of ganglion cells present and an increase in apoptosis mostly in the presumptive ganglion cell layer (GCL). However, Hh inhibition increases the number of ganglion cells but does not have an effect on cell death. Taken together, our results suggest that the hedgehog pathway is an important modulator of retina regeneration.

Supplemental data available online

Key words: Retina, Regeneration, Hedgehog, Fgf, Transdifferentiation

Introduction

Few organisms have the capacity to regenerate the retina. Examples of those that are able to undergo this remarkable process include fish, urodele amphibians and birds (for reviews, see Raymond and Hitchcock, 1997; Raymond and Hitchcock, 2000; Reh and Fischer, 2001; Del Rio-Tsonis and Tsonis, 2003; Haynes and Del Rio-Tsonis, 2004; Hitchcock et al., 2004; Tsonis and Del Rio-Tsonis, 2004).

It has been demonstrated that postnatal chickens have embryonic-like retinal stem cells located in the ciliary body (CB) and retina progenitor cells in the ciliary marginal zone (CMZ) that are able to generate some retinal neurons. The retinal stem cells in the ciliary body of the adult chicken only proliferate in response to growth factors and the non-pigmented epithelium in the CB has been shown to proliferate and give rise to retinal neurons in response to injury (Fischer and Reh, 2000; Fischer and Reh, 2003) (for a review, see Haynes and Del Rio-Tsonis, 2004).

The focus of this report, however, is the embryonic chick, which also has the capability to regenerate its retina. Unlike the adult chicken, the chick can regenerate all of the retinal layers after they have been completely removed by retinectomy during a small window in their development around embryonic day 4 (E4; Hamburger and Hamilton stages 22-24.5) (Coulombre and Coulombre, 1965). Members of the Fgf family such as Fgf1 and 2, are required to induce retina regeneration after its removal. Other growth factors such as TGFβ, insulin, IGF1 and 2 and NGFβ do not promote regeneration (Park and Hollenberg, 1989; Park and Hollenberg, 1991).

Interestingly, the embryonic chick retina can regenerate by two distinct modes; via transdifferentiation of the retina pigmented epithelium (RPE), or via activation of cells found in the anterior marginal region of the eye, near the ciliary body (CB) and ciliary marginal zone (CMZ) (Coulombre and Coulombre, 1965). The CB consists of two layers of cells, the pigmented and non-pigmented ciliary epithelium (PE and CE, respectively). Because there are no molecular markers that can clearly distinguish between the CB and the CMZ during the early stages of development, we will refer to this region as the CB/CMZ.

During transdifferentiation, the developing RPE loses its characteristic phenotype, becoming depigmented and proliferates to form a neuroepithelial layer that then differentiates into the various layers of the retina. This Fgf2-stimulated transdifferentiation occurs primarily in the posterior part of the optic cup and gives rise to a retina with reverse polarity, where the outer nuclear layer (ONL) is located on the inner surface of the eye, and the ganglion cell layer (GCL) is on the outer surface of the eye. The transdifferentiated retina lacks the RPE layer, which does
not replenish itself during regeneration (Coulombre and Coulombre, 1965).

The other mode of regeneration that gives rise to a fully differentiated retina appears as a consequence of the activation of stem/progenitor cells in the CB/CMZ. These cells have been shown to have activity up to E9 (Willbold and Layer, 1992). The regenerated retina forms with normal polarity and can associate with adjacent RPE that did not undergo transdifferentiation (Coulombre and Coulombre, 1965; Park and Hollenberg, 1991). Until the present report, the only times both of the aforementioned embryonic modes of regeneration were observed together in the same eye was during the classic Coulombre and Coulombre studies (Coulombre and Coulombre, 1965) where a piece of retina was used as a source of Fgf, and when Park and Hollenberg (Park and Hollenberg, 1991) used Fgf1 as a stimulus. We show that Fgf2 is able to promote both types of regeneration.

All studies carried out to date dealing with retina regeneration in the embryonic chick have focused on identifying factors that are responsible for the induction of regeneration, and are based solely on histological observations during a limited number of time points. Here we present a comprehensive study of Fgf2-induced regeneration. We use several cell-specific markers to identify the different cell types that form during regeneration and compare the temporal and spatial nature of the two modes of retina regeneration with normal retina development. We also show for the first time that retina regenerating from the anterior region of the eye originates from retina stem/progenitor cells located in the CB/CMZ.

Along with studying the temporal and spatial nature of the regenerating chick retina, we were also interested in identifying how key molecules control or influence regeneration. Sonic hedgehog (Shh) has been shown to play an important role in the development of the retina in a number of different model organisms (Levine et al., 1997; Greenwood and Struhl, 1999; Neumann and Nusslein-Volhard, 2000; Stenkamp et al., 2000; Zhang and Yang, 2001a; Zhang and Yang, 2001b; Wang et al., 2002; Dakubo et al., 2003; Perron et al., 2003; Stenkamp and Frey, 2003; Shkumatava et al., 2004). Shh is of particular interest because it has been shown that ectopic Shh expression in developing chick eyes causes retina to transdifferentiate into RPE, and inhibition of the Shh pathway causes the RPE to transdifferentiate into neuroepithelium (Zhang and Yang, 2001a). Hedgehog signaling is also implicated in the specification of the proximodistal axis and differentiation of RPE in developing Xenopus embryos (Perron et al., 2003); it helps establish a proximodistal axis during zebrafish eye development (Takeuchi et al., 2003) and is required for proper lamination and organization of the retina (Wang et al., 2002; Shkumatava et al., 2004). This research points to Shh being involved during transdifferentiation of the regenerating chick retina. On a different note, Shh has been shown to regulate neural progenitor proliferation in vitro and in vivo (Lai et al., 2003). In addition, the downstream effectors of the hedgehog pathway, Smootherned (Smo), Gli2 and Gli3 are strongly expressed in retinal progenitor cells of the CMZ in Xenopus (Perron et al., 2003), suggesting that the hedgehog pathway may play a role in regeneration occurring from the CB/CMZ.

In the current study, we show that Shh acts in a similar way during retina regeneration as it does during development. We overexpressed Shh using anan Reas-Shh retrovirus and inhibited the Hedgehog (Hh) pathway using a potent synthetic and less toxic form of cyclopamine, KAAD (3-keto, N-amo-ethylaminocaproyl dihydrocinnamoyl). We demonstrate that an overexpression of Shh allows the RPE to maintain its phenotype and inhibits transdifferentiation normally seen under Fgf2 stimulation. In fact, almost all regeneration under these circumstances comes from the ciliary region, and only RPE that is closest to the source of Fgf2 transdifferentiates. Furthermore, we show, for the first time, that Shh alone is able to initiate regeneration from the CB/CMZ independently of Fgf2. Conversely, KAAD-mediated inhibition of the hedgehog pathway enhances the transdifferentiation of RPE into retina, with almost all of the RPE giving rise to new retina, and only the most anterior pigmented epithelium, including the ciliary body, not transdifferentiating. Our studies are in agreement with those of Zhang and Yang (Zhang and Yang, 2001b), demonstrating that overexpression of Shh causes a reduction in RGC production by negatively regulating undifferentiated ganglion progenitor cells, or controlling their cell population via cell death, while inhibition of the hedgehog signaling pathway increases RGC differentiation.

Our studies offer evidence that Shh regulates cells that are not fully committed, as demonstrated by transdifferentiation of the RPE, as well as being responsible for activation of retinal progenitor cells in the CB/CMZ.

Materials and methods

Chick embryos

White Leghorn chicken eggs were purchased from Berne Hi-Way Hatcheries, Berne, IN, USA and from the Ohio State University, Columbus, OH and incubated in a humidified rotating incubator at 38°C.

Preparation of Fgf2, KAAD and PD173074

Heparin-coated polyacrylamide beads (Sigma, St Louis, MO, USA) were washed three times in phosphate-buffered saline (PBS). Fgf2 (R&D Systems, Inc., Minneapolis, MN, USA) was resuspended in 1× PBS at a concentration of 1 µg/µl. Heparin beads were then incubated in Fgf2 for at least 2 hours before use. A 1 mM KAAD (Toronto Research Chemicals, Ontario, Canada) stock was prepared in 100% ethanol. Affi-gel Blue beads (BioRad, Hercules, CA, USA) were washed in PBS and dehydrated through a series of ethanol washes of increasing concentration. KAAD stock solution was added to the Affi-gel Blue beads to a final working concentration of 100 µM. The Fgf2 inhibitor PD173074 (a kind gift from Pfizer, New York, NY, USA), was resuspended in DMSO at a concentration of 100 mM and incubated in ethanol-dehydrated Affi-gel Blue beads.

Surgical procedures

A window was made in the egg using forceps and microsurgical removal of the retina was carried out as previously described, at about Hamburger and Hamilton stage 24 (Coulombre and Coulombre, 1965; Park and Hollenberg, 1989). Briefly, a fine tungsten wire was used to make an incision in the dorsal part of the eye. Microdissection scissors were then used to make a semi-circular cut so that access to the optic cup was possible via the dorsal portion of the anterior eye. Using fine forceps, the retina was teased loose, taking extra care not to damage the RPE. An Fgf2-coated heparin bead was placed into the eye. The eggs were covered with tape and placed back into the incubator until 1, 3, 5, 7 or 11 days after surgery at E5, E7, E9, E11 or E15, respectively, when the eyes were collected. The eyes were then...
processed for histology, immunohistochemistry, in situ hybridization or TUNEL assay.

**Tissue fixation and sectioning**

Tissues processed for histological sectioning were fixed in Bouin’s fixative for at least 24 hours and embedded in paraffin wax. Tissues used for immunohistochemistry were fixed in 4% formaldehyde, cryoprotected in 30% sucrose and embedded in OCT freezing medium (Sakura Finetek, Torrance, CA, USA). Tissues used for in situ hybridization were sectioned at 14 µm, whereas the ones used for in situ hybridization were sectioned at 14 µm.

**Antibodies**

The following antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. Anti-Pax6 1:10; anti-Napa 73 1:100; anti-visinin (7G4) 1:100; anti-BrdU (G3G4) 1:100; anti-AMV/3C2 1:100; anti-vimentin, 1:100. Anti-Brn3a, 1:100, was purchased from Covance Research Products, Inc. (Denver, PA, USA). Polyclonal p-ERK antibody (1:100) was purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Other antibodies were kind gifts: anti-Chx10, 1:1000 and anti-Shh, 1:1000, from Dr Thomas Jessell, Columbia University, New York, NY, USA and anti-Mif, 1:100, from Dr Makoto Mochii, Himeji Institute of Technology, Hyogo, Japan. Secondary antibodies include goat anti-mouse FITC, goat anti-rabbit Alexa 488, goat anti-mouse Alexa 546, goat anti-mouse Alexa 488 and streptavidin-conjugated Alexa Fluor 350 (Molecular Probes, Eugene, OR, USA).

**Immunohistochemistry**

A general immunohistochemical protocol was used. Frozen sections were washed in 1× PBS and blocked for 1 hour. When antibodies against transcription factors were used, a 5-minute 1% saponin (Sigma, St Louis, MO, USA) wash was used followed by three washes in 1× PBS. Primary antibodies were diluted in blocking solution and sections were incubated overnight either at room temperature or 4°C, followed by washes in 1× PBS and incubation with secondary antibodies for at least 1 hour. Coverslips were then placed on the slides using Vectashield mounting medium (Vector Labs, Burlingame, CA, USA).

**p-ERK immunohistochemistry**

Retinectomies were performed as described and collected 4 hours after an Fgf2-coated bead or control heparin bead was placed in the eye. Animals that received KAAD beads had these beads placed in their eyes for 2 hours prior to the Fgf2 beads. Animals that were infected with Rcas-Shh were given a subretinal injection of Rcas-Shh on E3.5 approximately 12-16 hours before the surgery. Four hours after the administration of the Fgf2, the embryos were processed for immunohistochemistry.

**In situ hybridization**

Shh and Ptc1 probes were kind gifts from Dr Cliff Tabin (Harvard University, Boston, MA, USA). All probes were prepared using a Dig RNA labeling kit (Roche Applied Sciences, Indianapolis, IN, USA) and hydrolyzed to 0.1 kb. Tissue sections were prepared as described above and in situ hybridization was performed using the manufacturer’s protocol with modifications (Roche Molecular Biochemicals: Nonradioactive In Situ Hybridization Application Manual, 2nd edition).

**Retroviral production**

Replication competent Rcas (A) retrovirus engineered to express Shh along with control Rcas construct expressing GFP were generous gifts from Dr Cliff Tabin (Harvard University; Boston, MA, USA), Dr Teri Belecky-Adams (IUPUI, Indianapolis, IN, USA) and Dr Ruben Adler (Johns Hopkins University, Baltimore, MD, USA). Retroviral stocks were prepared by transfecting cultured DF1 chicken fibroblasts (ATCC, Manassas, VA, USA) with retroviral DNA using lipofectamine reagent (Gibco Invitrogen Corp., Carlsbad, CA, USA). Retrovirus was collected and concentrated using a Millipore ultra collection device (Millipore, Billerica, MA, USA) and titered by diluting concentrated stocks and infecting cultured embryonic chicken fibroblasts following by immunohistochemistry using the AMV3C2 antibody against a viral coat protein to determine CFUs/µl (colony forming units/µl).

**Microinjection**

10^4-10^5 CFUs/µl of the Rcas-Shh or of control Rcas-GFP were mixed with Fast Green dye and injected into the eye cavity, after the retina had been removed, with a pulled glass capillary pipette using a mouth piece. Expression of virus was confirmed by immunohistochemistry.

**Quantitative real-time PCR**

Total RNA was isolated using the RNA II isolation kit (BD Biosciences, Palo Alto, CA, USA). 1.5 µg of RNA was then reverse transcribed using Improm II reverse transcriptase (Promega, Madison, WI, USA). Real-time PCR was carried out on a Rotor-Gene 3000 Thermocycler using Sybrgreen as a fluorophore (Molecular Probes, Eugene, OR, USA). For each sample, Ptc1 primers were used to determine Ptc1 levels and GapDH was used as an internal control. A dilution series of cDNA was used to run a standard curve for each primer set and for each sample in order to determine the amount of Ptc1 and GapDH cDNA in each sample. Once these levels were determined, the quantitative value of Ptc1 was divided by the value of GapDH for each sample in order to determine the relative amount of Ptc1 cDNA. RNA was isolated from nine eyes for each sample quantified. Each PCR reaction was carried out three times separately, in order to ensure accuracy of results. Statistics for the relative levels of Ptc1 cDNA include the average of the three separate PCRs normalized to the Fgf2-treated eyes. Student’s t-test was used to determine statistical significance.

**Ganglion cell counts**

Ganglion cells were detected using the Brn3a antibody at E11 with the immunohistochemical methods described above. Three separate eyes for each treatment group (KAAD/Fgf2, Fgf2 and Rcas-Shh/Fgf2) were used and ganglion cells were counted from 100 µm x 100 µm areas at random. The number of random areas counted for each experimental group were as follows: KAAD/Fgf2, n=56; Fgf2, n=96; Rcas-Shh/Fgf2, n=88. The counts from all square regions from all eyes in an experimental group were then used to determine the average number of ganglion cells per 10,000 µm² of regenerated retina. Student’s t-test was performed to assess statistical significance.

**Dil and BrdU labeling**

Dil cell labeling paste (Molecular Probes, Eugene, OR, USA) was used to track cells of the CB/CMZ during regeneration. Retinectomies were performed as described above. This exposed the lens and surrounding CB/CMZ region. Using a glass micropipette, Dil cell labeling paste was carefully transferred onto the surface of cells in the CB/CMZ area only. Embryos were collected either a few minutes after the surgery, to ensure that only cells in the CB/CMZ were labeled and that there was no transfer of Dil onto the RPE, or at E5 or E7 to track the cells that regenerate from the CB/CMZ. Regenerating eyes were labeled with BrdU (Roche, Indianapolis, IN, USA) by micropipetting 1 µl of 10 mM BrdU solution into the optic cup. The eyes were collected 1-3 days post-retinectomy and processed for immunohistochemistry as described above.
**Dil anterograde labeling to follow axon fibers**

Retinectomies were performed as described above and E11 regenerating and control eyes were fixed with 4% formaldehyde overnight. The eyes were then injected with 2-3 \( \mu l \) of Dil (Molecular Probes, Eugene, OR, USA). The embryos were incubated in PBS at 37°C for 10 days. The eyes were sectioned and Dil labeling was observed using confocal microscopy.

**TUNEL**

Cell death was detected by the TUNEL assay using the in situ cell death detection kit, TMR Red (Roche, Indianapolis, IN, USA) as per the manufacturer’s instruction. Three separate eyes for each treatment group (KAAD/Fgf2, Fgf2 and Rcas-Shh/Fgf2) were used and cell death in 100 \( \mu m \times 100 \mu m \) random regions was quantified. The number of random areas counted for each experimental group are as follows: KAAD/Fgf2, \( n = 59 \); Fgf2, \( n = 49 \); Rcas-Shh/Fgf2, \( n = 49 \). The counts from all square regions from all eyes in an experimental group were then used to determine the average cell death per 10,000 \( \mu m^2 \) of regenerated retina. Student’s \( t \)-test was performed to assess statistical significance.

**Results**

**Retina regeneration mirrors normal development spatially and temporally**

In order to examine characteristics of the regenerating retina, the entire retina was removed from stage 22-24.5 chick embryos (embryonic day 4, E4) and a heparin bead coated with Fgf2 was placed into the optic cup to induce regeneration (Fig. 1A). Control experiments using heparin beads alone do not promote regeneration (Fig. 1B). At different time points, 3, 5, 7 or 11 days after retinectomy (E7, 9, 11 and 15 respectively), eyes were collected for histological observations. At all stages examined, two different sources for regenerating retina were observed (Fig. 1C). Transdifferentiation of RPE into new retina

**Fig. 1.** Fgf2 induces retina regeneration in two distinct ways. (A) At E4 the retina is removed surgically leaving behind RPE and CB/CMZ. An Fgf2-soaked heparin bead (*) is then placed in the eye cup. (B) A heparin bead (not visible in this section) alone does not cause regeneration after 7 days (E11). (C) By E11, Fgf2 induces regeneration from the CB/CMZ (cr) and by the transdifferentiation of the RPE (td). (D-O) Histology of normally developing as well as regenerating retina at E7 (D-F), E9 (G-I), E11 (J-L) and E15 (M-O). D,G,J,M show normal development at each stage. Three days after retinectomy (E7) a transdifferentiated neuroepithelium (E) as well as a neuroepithelium generated from cells in the CB/CMZ (F) are present. At E9, the transdifferentiated neuroepithelium (H) as well as the neuroepithelium arising from the CB/CMZ (I) thicken and grow. (K,L) Seven days post-retinectomy, at E11, the various retinal layers become visible. By E15, regenerated retinas (N,O) are laminated and resemble an E11 developing retina (J). Scale bar: 100 \( \mu m \) (A,B); 500 \( \mu m \) (C); 100 \( \mu m \) (D-O). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; ne, neuroepithelium; l, lens; CB/CMZ, ciliary body/ciliary marginal zone; RPE, retinal pigmented epithelium.
was observed mostly in the posterior of the eye and regeneration of new retina from the anterior margin originated at the CB/CMZ and projected posteriorly (Fig. 1C). Regeneration of both types occurred in a similar temporal and spatial manner (compare Fig. 1E,H,K,N with F,I,L,O). Retinas undergoing transdifferentiation showed depigmentation of the RPE as early as 24 hours after removal of the retina and administration of Fgf2 (not shown), and by E7 (3 days post-retinectomy) a new neuroepithelium was seen where transdifferentiation had occurred (Fig. 1E). A neuroepithelium also formed from the CB/CMZ (Fig. 1F and Fig. 2). It was not until E11 that the regenerating retina started to become properly organized into the different retinal layers, similar to an E9-E11 retina during normal development (compare Fig. 1G,J with K,L). The different orientation of transdifferentiated and CB/CMZ regenerate is easily observed at this stage of regeneration. Both eyes in Fig. 1K and L are oriented the same way, with the lens being at the top. The ganglion cell layer is easily distinguishable in both figures, however in the transdifferentiated retina, it is closest to the former RPE, where the photoreceptors would be expected (Fig. 1K). Conversely in the CB/CMZ regenerate, the ganglion cell layer is oriented the same as during normal development (compare Fig. 1J and L). Finally, by E15, both transdifferentiated and CB/CMZ regenerated retina were organized in a similar manner to that of an E11-E15 developing retina, with the various nuclear and plexiform layers becoming easily visible.

**Stem/progenitor cells are responsible for regeneration from the CB/CMZ**

In order to confirm that regeneration is indeed initiated from the CB/CMZ, we determined that this region has actively dividing stem/progenitor cells 4 hours after retinectomy (Fig. 2A-C). Collagen type IX normally expressed in the anterior portion of the eye was used to mark the presumptive CB/CMZ (Kubo et al., 2003) (Fig. 2A). This region of the eye contains mitotically active cells that co-express Pax6 and Chx10, suggesting these progenitor cells are mitotically active (Belecky-Adams et al., 1997) (Fig. 2B,C). To show that these active stem/progenitor cells are responsible for the regenerated retina, we traced the cell movements by labeling the CB/CMZ with DiI immediately after removal of the retina, and confirmed that this area was labeled with collagen type IX by immunohistochemistry approximately 4 hours after the retinectomy was performed (Fig. 2D). Eyes were collected 1 and 3 days later (Fig. 2E and F, respectively). The regenerating retina was labeled with DiI, showing a high level of fluorescence closest to the CB/CMZ. This fluorescence faded towards the posterior of the optic cup, indicating that cells from the CB/CMZ had divided, giving rise to a regenerated retina. Repeated cell division in this tissue results in a dilution of the DiI, as the retina regenerates away from the source of the DiI in an anterior to posterior pattern. The presence of mitotically active Pax6- and Chx10-positive progenitor cells in this area, along with DiI tracing of regenerating retina from this area suggests that the new retina results from activation of stem/progenitor cells in the CB/CMZ.

**All retinal cell types are present in the regenerating retina**

To examine regeneration of all cell types, and their laminar organization within the regenerating retina, we used cell-specific markers for each of the cell types found in the retina, as well as the RPE and the nerve fiber layer (NFL). The following antibodies were used for double-labeling experiments: Napa73 and Brn3a to detect the NFL and GCL, respectively; Pax6 to detect horizontal, amacrine and ganglion...
cells along with Chx10 to detect bipolar cells. In addition, co-expression of these two cell markers identifies progenitor cells. Visinin and Mitf detect photoreceptors and RPE, respectively. Vimentin is used to identify Müller glia.

The E7 developing retina shows some differentiated ganglion cells and nerve fibers (Fig. 3A1), along with a photoreceptor layer that is not well defined (compare Fig. 3A3 with D3). At this stage in development, the INL and ONL are not completely defined, as shown by the disorganized cell layers in Fig. 3A2. Also at this time in development some of the cells in these layers of the retina still contain undifferentiated progenitor cells, as indicated by co-expression of Pax6 and Chx10, while other cells are undergoing cell specification and differentiation.

Müller Glia are uniformly distributed throughout the retina (Fig. 3A4). The regenerated retina that originates both from transdifferentiation (Fig. 3B1-B4) and from the CB/CMZ (Fig. 3C1-C4) at this stage shows no Brn3a expression, a marker for differentiated ganglion cells, however, there are some Napa73-expressing cells. These cells are probably ganglion cells that have not fully differentiated (Fig. 3B1,C1). Regeneration has produced a Pax6/Chx10 neuroepithelium, which presumably, has not yet become committed to differentiate into the retinal layers (Fig. 3B2,C2). Transdifferentiating retina shows visinin-positive cells in the presumptive photoreceptor layer and no Mitf expression (Fig. 3B3), consistent with downregulation of Mitf upon transdifferentiation (Mochii et al., 1998a; Mochii et al., 1998b). CB/CMZ regeneration shows strong Mitf expression in the RPE, but shows no photoreceptor differentiation at this point (Fig. 3C3). No obvious differences are observed in the organization of the glia (Fig. 3A4,B4,C4).

By E11 both transdifferentiated and CB/CMZ regenerated retinas appear similar to those of normal E11 development, suggesting that the regenerating retina is able to ‘catch up’ temporally to normal development (compare Fig. 3D1-D4 with 3E1-F4). Mitf expression is absent in the transdifferentiated retina since there is no RPE layer (Fig. 3E3), whereas
Fig. 3. Regeneration gives rise to all cells layers of the retina. Rows: de, normal retinal development; td, retinas that arose from transdifferentiation; cr, retinas that regenerated from the CB/CMZ. Immunohistochemistry using antibodies against Brn3a (ganglion cells) and Napa73 (NFL) are shown in the first column (A1-I1). Immunohistochemistry using antibodies against Pax6 (ganglion cells, amacrine cells and horizontal cells) and Chx10 (bipolar cells) are shown in column 2 (A2-I2). Immunohistochemistry using antibodies against visinin (photoreceptors) and Mitf (RPE) are shown in column 3 (A3-I3). Sections in column 4 (A4-I4) were stained for vimentin to detect Müller glia. Rows A, B and C show eyes at E7, D, E and F at E11 and G, H and I at E15. (Row A) At E7, during eye development, ganglion cells are starting to differentiate and an NFL is visible (A1). Pax6 is mostly expressed in the region where ganglion cells are present while Chx10 is expressed through a large part of the retina (A2). Photoreceptors have started to differentiate and Mitf expression in the RPE is noticeable (A3). At this stage Müller glia are also present (A4). (Row B) At E7 transdifferentiating retina shows some Napa73 staining but no Brn3a (B1). Pax6 is expressed throughout the retina but its expression is very prominent in the ganglion cells. There are low levels of Chx10 at this stage (B2). There is no detectable Mitf expression in the retina that arises from transdifferentiation and visinin is expressed in the presumptive photoreceptor region. (B3). Vimentin expression is limited to Müller glia that span the retina (B4). (Row C) Retina that regenerates from the CB/CMZ shows a similar pattern of cell markers as the retina that arises from transdifferentiation. There is no Brn3a expression but there is some Napa73 staining (C1), and Pax6 is expressed throughout the retina, while there is a very low level of Chx10 expression (C2). However, visinin is not present and Mitf is expressed in the intact RPE (C3). Müller glia are present at this stage (C4). (Row D) By E11, developing retina has a clearly defined NFL, GCL (D1), INL (D2) and ONL (D3). Mitf continues to be expressed in the RPE (D3) and Müller glia are present throughout the retina (D4). (Row E) Transdifferentiation results in all differentiated cell types by E11. Ganglion cells (E1), amacrine cells, bipolar cells, horizontal cells (E2), photoreceptors (E3) and Müller glia (E4) are present. The orientation of this layer, however, is flipped (E1-E4). (Row F) Regeneration from the CB/CMZ also results in production of all differentiated cell types by E11. The NFL, GCL (F1), INL (F2), and ONL (F3) have all the cells types seen in a normal retina. Here, the RPE is still intact and expresses Mitf (F3). Müller glia span the retina (F4). The orientation of this layer is similar to that of developing retina. These expression patterns continue to be maintained at E15 in the developing eye (G1-G4), the transdifferentiated retina (H1-H4) and the retina that regenerated from the CB/CMZ (I1-I4). Scale bar: 100 μm.

Mitf is expressed in areas where the retina regenerates via the CB/CMZ (Fig. 3F3). Müller glia patterning resembles normal development (Fig. 3D4,E4,F4). At E15 all cell layers are present and organized in both regenerates (Fig. 3G1-I4).

Whole-mount eyes were prepared according to the method of Fischer and Reh (Fischer and Reh, 2003) and analyzed for patterns of regenerative activity in all four quadrants (dorsal-ventral, nasal-temporal) with no obvious differences (not shown). We also examined the E11 regenerating eyes to determine if the regenerating retina was forming an optic nerve. Using DiI anterograde labeling we found that the regenerating retina was sending projections out of the eye, however, these projections were very disorganized and did not resemble a normal optic nerve (Supplemental Fig. S1, http://dev.biologists.org/cgi/content/full/131/18/4607/DC1).

Hh patterns are unchanged during regeneration
Because Shh plays many important roles during normal eye development, from patterning to differentiation, we looked at the expression of Shh and its receptor, Patched 1 (Ptc1) during normal retina development and during regeneration.

In situ hybridization studies show that during normal development (at E11) Shh expression is localized to the ganglion cell layer, whereas Ptc1 expression is found in part of the INL and in the ONL in the posterior retina (Fig. 4A1,A3). Immunohistochemistry studies show that Shh protein is present at high concentration closer to the ganglion cells, and is diffused throughout the retina in a gradient-like fashion (Fig. 4A2). In the anterior region of the eye, where the CB/CMZ is located, Shh mRNA is expressed (Fig. 4B1) along with Ptc1 (Fig. 4B3). Shh protein is also found in the CB/CMZ (Fig. 4B2).

Shh mRNA and protein expression were very low in the ganglion cells of the CB/CMZ regenerated retina (Fig. 4C1,C2). Ptc1 expression, however, was similar to that in the developing retina (Fig. 4C3). CB/CMZ regenerated retina in the anterior portion of the eye (CB/CMZ region) had Shh and Ptc1 expression patterns that were similar to those seen during normal development (compare Fig. 4B1,B2,B3 with Fig. 4D1,D2,D3).

Transdifferentiated retina had similar expression patterns to that of developing retina, with Shh message detectable in the presumptive GCL, and Ptc1 message mostly present in the INL and ONL (compare Fig. 4E1,E3 with A1,A3). Shh protein was also observed in a gradient, in which the expression was strongest close to the GCL (Fig. 4E2).

Function of Shh during retina regeneration
In order to determine a possible role for Shh during retina regeneration, we overexpressed Shh using a retrovirus, Rcas-Shh, or inhibited the hedgehog pathway using KAAD.

Shh overexpression was confirmed using in situ hybridization for the Shh transcript (Fig. 5A1) as well as by assaying the changes in Ptc1 expression, a downstream effector of the Hh pathway (Fig. 5A3). To confirm that ectopic Shh transcript was being translated into protein, we performed immunohistochemical studies using an antibody against Shh (Fig. 5A5). Both the in situ hybridization and the immunohistochemistry show similar ectopic expression patterns of Shh that are characterized by heavy ‘patches’ of expression throughout the retina. We also confirmed infection of the virus using the AMV 3C2 antibody against a viral coat protein (Fig. 5A6). This method of detecting viral infection was also indicated by patches of immunopositive retina.

Since in situ hybridization is not an accurate quantitative assay, we used real-time PCR to show that we were effectively inhibiting or activating the Hh pathway with our treatments. It is known that an increase in Shh will result in an increase Ptc1 expression (Ingram et al., 2002) and inhibition of the Hh pathway will lead to a decrease in Ptc1 expression (Chiang et al., 1999; Mill et al., 2003; Tsonis et al., 2004). Our PCR data confirms that the treatments were effective, as Ptc1 expression was significantly increased in regenerating eyes treated with the Rcas-Shh retrovirus (P<0.01), and significantly decreased in regenerating eyes treated with KAAD (P<0.05) (Fig. 5B) when compared to Ptc1 expression in Fgf2-treated eyes.
Shh plays a role in RPE maintenance during regeneration

Eyes were treated with Fgf2 alone, Rcas-Shh/Fgf2 or KAAD/Fgf2 and examined at E7, E11 or E15 (Fig. 6). There were no obvious differences in laminar organization between the three treatments. At E7, all three treatments gave rise to a neuroepithelium (Fig. 6B,C,E,F,H,I). By E11, regenerating retina from the CB/CMZ in the KAAD/Fgf2- and Fgf2-treated eyes had a GCL, IPL and INL, however, the OPL and ONL had not yet differentiated (Fig. 6K,N). The Rcas-Shh-treated eyes appeared to be more differentiated as the OPL and ONL were visible (Fig. 6Q). In contrast, the transdifferentiated retina in all treatment groups had all retinal layers present (Fig. 6L,O,R). All layers were well defined at E15 (Fig. 6T,U,W,X,Z). Although there were no obvious defects in cellular location and organization during Shh manipulation, eyes treated with KAAD/Fgf2 after retinectomy at stage 24 showed an increased domain in transdifferentiation from the posterior towards the anterior when compared to the Fgf2-treated eyes (compare Fig. 6A,J,S with D,M,V). It appears that in the KAAD-treated eyes, only the anterior-most pigmented cells, including the pigmented ciliary epithelium, remained untransdifferentiated (Fig. 6A,J,S). Eyes treated with KAAD alone showed no regeneration from the CB/CMZ or from transdifferentiation (not shown).

Rcas-Shh/Fgf2-treated eyes show the opposite phenotype to that of the KAAD/Fgf2-treated eyes and those treated with Fgf2 only. Transdifferentiation was observed in very few of the Rcas-Shh/Fgf2-treated eyes (compare Fig. 6G,Y with P). The majority of regeneration came from the CB/CMZ, and transdifferentiation was not observed in any cases at E15 (Fig. 6Y). These histological results indicate that the Shh pathway may play a role in the maintenance of the RPE, inhibiting transdifferentiation, as well as promoting proliferation and regeneration from the CB/CMZ.

In order to determine if the Shh pathway is playing a similar role during normal development, E3.5 eyes were injected with Rcas-Shh subretinally so that the viral infection was localized between the retina and RPE. Eyes were given approximately 12 hours for the virus to be expressed. At E4, an incision was made in the extraocular mesenchyme behind the RPE and an Fgf2-soaked bead was placed next to the RPE in both Rcas-Shh-injected eyes and control eyes that received no Rcas virus. When the eyes were examined two days later, 80% (n=5) of the Fgf2-treated eyes showed some transdifferentiation, whereas 80% (n=5) of control eyes showed some transdifferentiation, whereas the Rcas-Shh-treated eyes showed no transdifferentiation (n=6; Fig. 7A).

![Fig. 4. Shh and Ptc1 expression in E11 eyes. (A1-B3) Developing retina (de) at E11. (C1-D3) Retina regenerating from the CB/CMZ (cr). (E1-E3) Transdifferentiating retina (td) at E11. (A1) Expression of Shh mRNA during normal development is predominantly in the ganglion cells of the posterior retina. (A2) Shh protein expression in the posterior retina of the developing eye mirrors that of the mRNA expression. (A3) Ptc1 mRNA expression in the posterior retina of a developing eye is located through the INL and ONL. (B1) Shh mRNA expression in the anterior part of the developing eye includes the presumptive CMZ. (B2) Shh protein expression is similar to that of the mRNA expression. (B3) Expression of Ptc1 mRNA is in the anterior retina and ciliary region. (C1,D1) During CB/CMZ regeneration, Shh expression patterns basically remained unchanged, however the levels in the posterior region were low (arrowhead in C1). (C2,D2) Shh protein expression of retina regenerating from the CB/CMZ. (C3,D3) Ptc1 expression is present throughout part of the INL and ONL in the posterior (arrowhead in C3) as well as anterior part (arrowhead in D3) of the CB/CMZ regenerated retina. (E1) Transdifferentiating retina shows Shh mRNA expression in the ganglion cell layer (arrowhead). (E2) Shh protein expression is diffused throughout the transdifferentiating retina but the expression is mainly in the ganglion cell layer (arrowhead). (E3) Ptc1 expression is predominantly in the INL of the transdifferentiated retina (arrowhead). F1, F2 and F3 are negative controls for Shh RNA, Shh Protein and Ptc1 RNA expression respectively. Arrowheads mark areas of expression.](image-url)
Hh, Fgf and transdifferentiation

We wanted to know whether overexpression of Shh or inhibition of the Hh pathway is involved in regulating the amount of transdifferentiation by exerting some effect on the Fgf pathway. In order to do this, retinectomies were performed at E4 and the eyes given one of four possible treatments: (1) no Fgf2 bead; (2) Fgf2 bead; (3) Fgf2 bead plus KAAD beads; (4) subretinal injection of Rcas-Shh at E3.5 followed by Fgf2 bead at E4.

Eyes were collected 4 hours later and immunostained for the active form of ERK, phospho-ERK. ERK is activated through the Fgf pathway, thus ERK phosphorylation can be used to determine Fgf signaling activity. By examining phospho-ERK activation it is evident that the Fgf pathway is being stimulated by Fgf2, compared to the control, which did not have an Fgf2 bead (Fig. 7B). In eyes treated with KAAD and Fgf2, phospho-ERK immunoreactivity was similar to levels of phospho-ERK immunoreactivity with Fgf2 alone. It is very noticeable, however, that overexpression of Shh inhibits ERK phosphorylation, as almost no immunoreactivity was detected, suggesting it is having a negative effect on Fgf signaling (Fig. 7B).

These results indicate that the Hh pathway is involved in RPE maintenance by regulating Fgf-stimulated signaling upstream of ERK in the RPE.

Shh activates stem/progenitor cells in the CB/CMZ via a Fgfr signaling pathway

Interestingly, we observed regeneration from the CB/CMZ region when Shh was overexpressed and ectopic Fgf2 was absent. In 60% of eyes treated with Rcas-Shh alone (n=15) there was robust regeneration from the CB/CMZ (not shown; similar to that seen in Fig. 6G in one set of experiments and 89% in another set (n=9; Fig. 7C), and no regeneration via transdifferentiation. Variation in infectivity may help to explain why only 60%-89% of Rcas-Shh-treated eyes were able to regenerate a retina.

To ensure that the Rcas virus was not contributing to regeneration, we infected eyes with Rcas-GFP after removing the retina. These eyes showed no regeneration (not shown).

Since we observed that Hh modulates the Fgf signaling pathway in the RPE, and that Shh is sufficient to promote regeneration in the CB/CMZ, we wanted to see if the Hh pathway was working through a Fgf pathway in the CB/CMZ. To do this, we removed the retina from eyes at E4 and injected Rcas-Shh and added PD173074, a potent Fgfr antagonist (Bansal et al., 2003). As a control to ensure that PD173074 was having the desired effect of antagonizing the Fgfr, we removed the retina at E4 and added Fgf2 + PD173074. Controls were done for both experiments substituting DMSO for PD173074. Eyes were allowed to regenerate for 3 days before being processed for histology and assayed for regeneration. Eyes treated with Fgf2 + PD173074 showed a significant decrease in regeneration compared with controls (\(\chi^2, P<0.025\)), as only 33% (n=11) had regenerating retina, indicating that PD173074 was efficiently antagonizing Fgfr and inhibiting Fgf signaling. Rcas-Shh + PD173074 eyes also showed a significant decrease compared with controls, with only 30% of eyes showing regeneration from the CB/CMZ (n=10) (\(\chi^2, P<0.01\)). Rcas-Shh control eyes (with DMSO) showed approximately 89% regeneration from the CB/CMZ (n=9) and Fgf2 control eyes (with DMSO) showed 100% regeneration from the CB/CMZ (n=4) (Fig. 7C).

Shh controls ganglion cell populations during retina regeneration

When analyzing these treated eyes for retinal cell markers, all three treatment groups, Rcas-Shh/Fgf2, KAAD/Fgf2 or Fgf2 alone, had patterns similar to those of Fgf2-treated eyes at E7 (Fig. 3B1-C4), having a mostly undifferentiated neuroepithelium (data not shown). By E11 and E15, both Rcas-Shh/Fgf2 and KAAD/Fgf2-treated eyes also resembled Fgf2-treated eyes (Fig. 8A1-K3): all eyes were positive for cell markers for NFL, GCL, amacrine cells, bipolar cells, horizontal cells, photoreceptors and RPE, (the RPE is not present in panels showing transdifferentiation; Fig. 8D3,E3,F3,J3,K3). One recognizable difference between Rcas-Shh/Fgf2 and KAAD/Fgf2 treatments was that the KAAD/Fgf2-treated eyes at E11 appeared to have more...
ganglion cells than the Fgf2 eyes and Rcas-Shh/Fgf2-treated eyes appeared to have fewer ganglion cells than the Fgf2 only-treated eyes (compare Fig. 8A1,D1,B1,E1 and C1,F1).

To confirm that inhibiting the Hh pathway or ectopic expression of Shh affects the differentiation of ganglion cells in the regenerating eye, we counted the number of ganglion cells per 10,000 \( \mu m^2 \) in eyes treated with Fgf2 alone, Rcas-Shh/Fgf2 and KAAD/Fgf2. Fgf2-treated eyes had an average of 7.168 ganglion cells/10,000 \( \mu m^2 \) of regenerated retina, whereas KAAD/Fgf2-treated eyes had significantly more, 8.821 ganglion cells/10,000 \( \mu m^2 \) (\( P<0.001 \)) (a 23.09% increase), and Rcas-Shh/Fgf2-treated eyes had significantly fewer, 4.09 ganglion cells/10,000 \( \mu m^2 \) (\( P<0.0001 \)) (a 42.98% decrease) in the regenerated retina (Fig. 9A). Therefore, along with maintenance and specification of the RPE, and the activation of cells in the CB/CMZ, Shh also plays a role in controlling the number of ganglion cells in the regenerating retina.

Fig. 6. Effect of Shh on regeneration. Inhibition of the Shh pathway using KAAD increases the domain of transdifferentiation (A-C,J-L,S-U) compared to eyes that received Fgf2 alone (D-F,M-O,V-X). However, overexpression of Shh using Rcas-Shh decreases transdifferentiation (G-LP-R,YZ). (A-I) E7. (A) At E7, KAAD/Fgf2-treated eyes show regeneration from the CB/CMZ (cr) as well the transdifferentiation of RPE (td). (B) A close up view of CB/CMZ from an E7 KAAD/Fgf2-treated eye. (C) A close up view of transdifferentiation from an E7 KAAD/Fgf2-treated eye. (D) Regenerating eye that has been treated with Fgf2 only. (E) A close up view of CB/CMZ regenerated retina. (F) Close up view of transdifferentiated retina. (G) Regenerating eye that has been treated with Rcas-Shh/Fgf2 shows almost no transdifferentiation. (H) CB/CMZ regenerated retina, where Shh has been overexpressed does not look different from that of E7 Fgf2-treated regenerating neuroepithelium (E). (I) Occasionally, there are small areas of transdifferentiation in the regenerating eyes that received Rcas/Shh/Fgf2. The neuroepithelium at this stage is similar to that of an Fgf2-treated transdifferentiating retina (F). (J-R) E11. (J) At E11, KAAD/Fgf2-treated eyes show an increased domain of transdifferentiation when compared to Fgf2-treated eyes of the same stage (M). (P) At this stage, several eyes that overexpress Shh show some transdifferentiation. KAAD/Fgf2 and Fgf2-treated eyes show similar organization having a GCL, IPL and INL (K,L,N,O); whereas Rcas-Shh/Fgf2-treated eyes seem to be more differentiated with a visible OPL and ONL (Q,R). (S-Z) E15. (S) At E15, most of the retina in KAAD/Fgf2-treated eyes results from transdifferentiation. However, there exist small regions that contain CB/CMZ regenerated retina. (V) Eyes treated with Fgf2 show both transdifferentiated retina and CB/CMZ regenerated retina. (Y) Rcas-Shh/Fgf2-treated eyes do not show any transdifferentiated retina and all the retina present is produced by regeneration from the CB/CMZ. All the retinas at this stage show similar levels of spatial organization (T,U,W,X,Z). Scale bars: 500 \( \mu m \) (A,D,G,J,M,S); 100 \( \mu m \) and is applicable to B,C,E,F,H,I,K,L,N,O,Q,R,T,U,W,X,Z; in P is 500 \( \mu m \) and applies to Y; 500 \( \mu m \) (V). An asterisk marks an Fgf2-coated heparin bead.
Fig. 7. Shh maintains the RPE phenotype and its actions in the RPE and the CB/CMZ depend on Fgfr signaling. (A) Developing eyes were treated with Fgf2 at E4, or Rcas-Shh and Fgf2 at E3.5 and E4, respectively, and analyzed at E6. Ectopic Shh was sufficient to inhibit transdifferentiation in developing eyes. B, Fgf2 bead; t, transdifferentiated retina. Arrowhead indicates developing retina. (B) Fgfr signaling activity was measured 4 hours after treatment by immunohistochemistry using a phospho-ERK antibody in retinectomized eyes with: no Fgf2, Fgf2, Fgf2/KAAD or Fgf2/Rcas-Shh. Fgf2 activates the Fgfr signaling pathway, whereas ectopic Shh inhibits this activity. Scale bar: 50 µm. (C) Retinectomies were performed at E4 and eyes were treated with Fgf2±PD 173074, or with Rcas-Shh±PD 173074, and analyzed 3 days later. Ectopic Shh stimulates regeneration from the CB/CMZ, as does Fgf2. Both of these activities are significantly inhibited when PD 173074, a potent and specific Fgfr inhibitor, is used.

Fig. 8. Effects of Shh on organization of the regenerating retina. Eyes that were treated with KAAD/Fgf2 (A,D,G,J), Fgf2 only (B,E,H,K) or Rcas-Shh/Fgf2 (C,F,I) were collected at E11 (A-F) or E15 (G-K) and were subjected to immunohistochemical analysis. Brn3a and Napa-73 immunoreactivity in E11 KAAD/Fgf2-treated eyes suggests that there are more ganglion cells and nerve fibers in both the CB/CMZ regeneration (A1) and retina that arises from transdifferentiation of the RPE (D1) when compared to the Fgf2-treated controls in (B1) and (E1) respectively. When eyes were treated with Rcas-Shh/Fgf2, regeneration from the CB/CMZ (C1) and from transdifferentiation (F1) seem to have a reduced NFL and number of ganglion cells. There seem to be no difference in the expression patterns of Pax6 and Chx10 in KAAD/Fgf2 (A2,D2) or Rcas-Shh /Fgf2 (C2,F2) when compared with the Fgf2-treated controls (B2,E2, respectively). There are no major differences in the expression of visinin and Mitf in the CB/CMZ regenerate when comparing KAAD/Fgf2-treated eyes (A1) with Fgf2-treated eyes (B1) and Rcas-Shh/Fgf2-treated (C1) eyes. There is also no difference in the expression of visinin in retina that regenerated by transdifferentiation, when we compare KAAD/Fgf2-treated (D3) with Fgf2 (E3) and Rcas-Shh/Fgf2-treated (F3) eyes. Similar patterns are observed at E15. Expression patterns of Pax6 and Chx10 are comparable among KAAD/Fgf2-treated (G2 and J2), Fgf2 only (H2 and K2) and Rcas-Shh/Fgf2-treated (I2) eyes. At E15, visinin is present while Mitf is absent in all transdifferentiated retina that arises in KAAD/Fgf2-treated eyes (J3) and control (K3) eyes. Scale bar: 50 µm.
To determine if the differences in the number of ganglion cells in the different treatment groups was due to cell death, we performed a TUNEL assay. There was a significant increase in cell death in the Rcas-Shh-treated eyes in both the total retina (Fig. 9B) and GCL (Fig. 9C) compared to Fgf2-treated eyes \((P<0.001)\). There was no significant difference in the number of apoptotic cells between KAAD- and Fgf2-treated eyes.

Discussion

We have described, for the first time, a detailed histological and cellular analysis of two unique types of retina regeneration in the embryonic chick as well as investigating the role of Shh in this process.

Transdifferentiation occurs when the RPE loses its characteristic pigmented phenotype, dedifferentiates, proliferates and forms a neuroepithelium that eventually gives rise to all of the layers of the neural retina. However, once the RPE undergoes transdifferentiation it is lost in that area of the eye and does not replenish itself. In vivo transdifferentiation does not take place after about E4.5 (Coulombre and Coulombre, 1965) (data not shown). It has been well documented that signals from both the surface ectoderm during eye induction and signals from the extraocular mesenchyme are required for proper development of the RPE and neural retina (Nguyen et al., 2000; Fuhrmann et al., 1998b). Specifically, there is a trend in the RPE for lower levels of retinaldehyde dehydrogenase type 1 (Raldh1) expression in the developing chick eye. This transdifferentiation is correlated with a characteristic pigmented phenotype in RPE cells, as well as rendering the RPE cells unable to respond to Fgf2 in culture (Mochii et al., 1998a). Early in eye development, when the optic cup is differentiating into retina and RPE domains, Fgf1 and Fgf2 signals from the surface ectoderm are required (Hyer et al., 1998; Pittack et al., 1997) for proper segmentation of retina and RPE. Studies on Fgf receptor type 1 (Fgfr1) and Fgf receptor type 2 (Fgfr2) expression in the developing chick eye show that the retina has the highest levels of these receptors at around E4, with levels dropping off until about E18. Specifically, there is a trend in the RPE for lower levels of Fgf receptor expression after E4. Expression that remains in the eye after E4 tends to be highest in the ganglion cells and photoreceptors (Tcheng et al., 1994; Ohuchi et al., 1994). Reduction of Fgf receptor expression in the RPE may help explain why the RPE loses its ability to respond to Fgf2 and to transdifferentiate in vivo after E4.5. It may be that since the RPE has the ability to transdifferentiate in vivo until E4.5, there is a transitional stage at which RPE must be responsive to the Fgf signals from the surface ectoderm for proper development, while at the same time the RPE starts to lose this responsiveness in order to become a fully committed and differentiated RPE. It has also been reported that constitutive activation of the Fgf downstream signaling molecule, MEK1, activates the MAPK signal transduction molecule ERK and induces transdifferentiation of RPE to retina in developing chick eyes. This transdifferentiation is correlated with a downregulation of Mitf in the RPE caused by proteolysis stimulated by ERK phosphorylation of Mitf (Galy et al., 2002). Our findings directly link Fgf signaling and regulation of RPE-specific factors, and indicate that loss of Fgf responsiveness may be required for the RPE to fully differentiate. In addition, Mochii et al. (Mochii et al., 1998a) indicated that levels of Mitf are not solely responsible for regulation of transdifferentiation. They show that there are higher concentrations of Mitf present in the RPE at E5 than...
E9. However, E5 RPE is able to transdifferentiate in vitro after only a short period in culture, whereas E9 RPE takes much longer. They suggest that the ability to transdifferentiate may be regulated by the sensitivity of cells to extracellular signals.

**A role for Hedgehog in RPE specification and differentiation in the regenerating retina**

Our results indicate that the Hh pathway plays an important role in the differentiation of the RPE. Inhibition of the Hh pathway using the potent teratogen KAAD causes the transdifferentiation domain in the regenerating eye to expand from the posterior to the anterior of the eye cup so that in most cases only the anterior-most pigmented epithelium remains intact. Additionally, ectopic expression of Shh inhibits transdifferentiation of the RPE into neural retina (NR), and only in a few cases did we observe transdifferentiation in these eyes. The transdifferentiation we did observe in Rcas-Shh/Fgf2-treated eyes occurred in an Fgf2 concentration-dependent manner, as only RPE closely associated with the source of Fgf2 transdifferentiated, most likely because these areas were in contact with the highest concentration of Fgf. We also show that the RPE maintenance role of Shh occurs during normal development. Our results support observations by Perron et al. (Perron et al., 2003), indicating that in *Xenopus*, Hedgehog signaling plays a clear role in proximodistal axis specification and RPE differentiation, and that inhibition of Hh signaling with cyclopamine causes RPE differentiation defects.

The transcription factor Otx2 is able to regulate RPE differentiation independently of Mitf, but has also been shown to bind and transactivate Mitf (Martinez-Morales et al., 2003). Zhang and Yang (Zhang and Yang, 2001a) have shown that ectopic Shh expression is able to cause misexpression of Otx2. It is possible that downregulation of Otx2 by inhibiting the Hh pathway is subsequently causing a loss of transactivation of Mitf, leading to its downregulation. In addition, activation of the Fgf pathway may be causing degradation of the Mitf protein. Combining this, with the indirect Hh downregulation of Mitf transactivation may have an additive effect for reducing Mitf protein and mRNA levels, explaining the enhanced transdifferentiation domain observed in our experiments. Although further studies are needed, our results suggest that the hedgehog pathway may play a role in retina regeneration by negatively interacting with the Fgf pathway to inhibit transdifferentiation, as is seen by our phospho-ERK immunostaining, where Rcas-Shh decreased ERK activation.

The connection between the Hh pathway and Mitf is seen in *Xenopus*, where it has been shown that inhibiting the Hh pathway also leads to a decrease in Mitf expression in the RPE (Perron et al., 2003). However in that study there was no correlation between Otx2 regulation and Mitf expression. Other studies have shown that Otx2 is negatively regulated by Fgf8 during development of the optic vesicle (Crossley et al., 2001), and that when a bead soaked with Fgf8 is implanted into the extraocular mesenchyme of a developing chick eye, it will induce transdifferentiation of the RPE into retina (Vogel-Hopker, 2000). There is also a possibility that a downstream target of Shh signaling may play a more direct role in regulation of the Fgf signaling pathway.

**Shh induced regeneration from the CB/CMZ is dependent on the Fgf signaling pathway**

A specific role for Shh in stimulating and enhancing regeneration from the CB/CMZ still needs to be elucidated. However, from our studies, it is clear that Shh overexpression is sufficient to cause stem/progenitor cells to give rise to a regenerated retina through the Fgf pathway. Our results show that Rcas-Shh promoted regeneration from the CB/CMZ in 60% and 89% (two different experiments) of eyes in the absence of Fgf2. It has been observed that Hh can act through the Fgf pathway in different contexts. For example, Fgf2 or Shh promote necortical precursors to differentiate into oligodendrocyte progenitors in culture. Fgf2-induced differentiation of necortical precursors is independent of the Hh pathway. However, Hh-stimulated differentiation is dependent on constitutive activity of Fgfrs, which maintain a basal level of active MAPK. Inhibition of Fgfrs in these necortical precursors inhibits Shh-stimulated differentiation into oligodendrocyte precursors (Kessaris et al., 2004). Our experiments inhibiting Fgfrs using the potent Fgfr antagonist PD173074 show that the mitogenic activity stimulated by Shh in the CB/CMZ is dependent on Fgf receptor signaling, and may require basal Fgfr activation, as seen in the Kessaris et al. study. Further experiments are needed to dissect the mechanism by which Shh acts on the Fgf pathway to exert its effects.

**Shh is sufficient to activate stem/progenitor cells in the CB/CMZ**

Combined, our results indicate that Shh has at least two different functions within the eye. The first is in RPE maintenance and is tied to the Fgf pathway. The second is in regulating and stimulating regeneration from the CB/CMZ. It has been demonstrated in *Xenopus* that Smoothered, Gli2 and Gli3, downstream targets of the Shh pathway, are highly expressed in stem/progenitor cells located in the CMZ and peripheral pigmented epithelium, implicating the hedgehog pathway in stem/progenitor cell maintenance (Perron et al., 2003). In addition, recent studies in the post-hatch chick show that Shh is present in the CMZ and that this region responds to ectopic Shh by proliferating (T. A. Reh, personal communication). Also, mice with a single functional allele of Ptc1 develop a CMZ-like structure not present in wild-type mice. This CMZ-like region expresses genes present in the CMZ of lower vertebrates and responds to injury by proliferating and giving rise to retinal neurons (Moshiri and Reh, 2004). These studies, in combination with the results presented here, indicate that the Hh pathway is essential for CB/CMZ development and maintenance and is probably involved in proliferation and generation of new neurons from this area.

**A role for Hedgehog in regulation of ganglion cells, during retina regeneration**

It is known that Hedgehog signaling is responsible for driving a wave of retinal differentiation in a variety of species. *Drosophila* Hedgehog plays a crucial role in patterning the compound eye (Greenwood and Struhl, 1999), and vertebrates including zebrafish and chick require waves of Shh expression for proper differentiation of retinal cell types (Neumann and Nuesslein-Volhard, 2000; Stenkamp et al., 2000; Zhang and...
Yang, 2001b; Stenkamp and Frey, 2003; Shkumatava et al., 2004). Specifically, Zhang and Yang (Zhang and Yang, 2001b) have shown in the developing chick retina that Shh is required for proper ganglion cell differentiation. Shh is needed to drive the differentiation wave of ganglion cells from the posterior to the anterior, while at the same time, ganglion cells that have already differentiated secrete Shh behind the wave of differentiation in order to inhibit surrounding progenitor cells from differentiating into more ganglion cells. Differentiation of progenitor cells into ganglion cells is inhibited if levels of Shh are ectopically increased, and ganglion cell production is increased if Shh levels are decreased or if the Hh pathway is inhibited (Zhang and Yang, 2001b). Our results support these previous studies indicating that overexpression of Shh inhibits ganglion cell production in the regenerating retina, whereas inhibition of the Hh pathway increases ganglion cell number.

Our tunnel assay results show that the decreased number of ganglion cells in the Rcas-Shh-treated eyes are, in part, due to increased cell death. It has been shown that Shh is able to inhibit progenitor cells from differentiating into ganglion cells (Zhang and Yang, 2001b). One possible reason for the increased cell death is that cells that do not differentiate may receive intrinsic or extrinsic cues to undergo apoptosis. It is unlikely that the ectopic expression of Shh is directly responsible for the increased apoptosis, since it has been shown that in the absence of a Shh signal, ectopic Ptc1 is able to induce apoptosis in the ventral spinal cord of embryonic chicks, and that a Shh signal is able to prevent the cell death from occurring (Thibert et al., 2003).

It has recently been suggested that ganglion cell production ends in a given region of the retina because of cell-cell interactions, and not because of loss of competence by progenitors to produce ganglion cells (Silva et al., 2003). Interestingly, in that study ganglion cell generation was observed to be complete by E11. Our studies showed that there is an increase in cell death in the Rcas-Shh regenerating eyes at E11, supporting the idea that there is some environmental cue that is responsible for initiating apoptosis in undifferentiated cells. In fact, Mayordomo et al. (Mayordomo et al., 2003) have shown that the ganglion cell population is modulated via a caspase 3-dependent cell death during early retina development. In addition, we saw no statistical difference between Fgf2- and KAAD-treated regenerating eyes, offering further evidence that the Hh pathway is not directly responsible for the apoptosis observed.

Conclusion

We demonstrate that retina regeneration by transdifferentiation as well as by the activation of stem/progenitor cells from the CB/CMZ can give rise to all the cell types of the retina. Furthermore, we show that Shh controls the domain of transdifferentiation and plays a role in the ability of RPE cells to transdifferentiate. This activity seems to be Fgf2 dependent. Shh is also able to induce regeneration from the CB/CMZ, which requires Fgfr activity, as antagonizing the receptor inhibits this type regeneration. Shh also seems to control the number of ganglion cells that are produced in the regenerated retina.

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References


