RESEARCH ARTICLE

Fibroblast Growth Factor–Hedgehog Interdependence During Retina Regeneration

Jason R. Spence, Juan-Carlos Aycinena, and Katia Del Rio-Tsonis*

The embryonic chick is able to regenerate the retina after it has been removed. We have previously shown that proliferating stem/progenitor cells present in the ciliary body/ciliary marginal zone (CB/CMZ) of the chick eye are responsible for regeneration, which can be induced by ectopic fibroblast growth factor-2 (FGF2) or Sonic hedgehog (Shh). Here, we reveal the mechanisms showing how FGF2 and Shh signaling are interdependent during retina regeneration. If the FGF pathway is inhibited, regeneration stimulated by Shh is inhibited. Likewise, if the Hedgehog pathway is inhibited, regeneration stimulated by FGF2 is inhibited. We examined early signaling events in the CB/CMZ and found that FGF2 or Shh induced a robust Erk phosphorylation during the early stages of retina regeneration. Shh also up-regulated the expression of several members of the FGF signaling pathway. We show that ectopic FGF2 or Shh overexpression increased the number of phosphohistone 3 (PH3)-positive cells in the CB/CMZ and inhibition of either pathway decreased the number of PH3-positive cells. Additionally, both FGF and Hh signaling are required for cell survival in the CB/CMZ, whereas Hh and not FGF signaling plays a role in maintaining the identity of the retinal progenitor population in this region. Combined, our results support a model where the FGF and Hedgehog pathways work together to stimulate retina regeneration. *Developmental Dynamics 236: 1161–1174, 2007.* © 2007 Wiley-Liss, Inc.

Key words: retina regeneration; CB/CMZ; sonic hedgehog; FGF; Erk; stem cell; progenitor cell

Accepted 1 February 2007

INTRODUCTION

The embryonic chick retina is able to regenerate during a window of early development (Coulombre and Coulombre, 1965, 1970; Park and Hollenberg, 1989, 1991; Spence et al., 2004) from two distinct sources (reviewed in Del Rio-Tsonis and Tsonis, 2003; Tsonis and Del Rio-Tsonis, 2004; Haynes and Del Rio-Tsonis, 2004; Vergara et al., 2005). One source of regeneration is by means of the process of transdifferentiation of the retina pigmented epithelium (RPE). This type of regeneration can occur until approximately embryonic day (E) 4.5 when an ectopic source of fibroblast growth factor (FGF) is present (Park and Hollenberg, 1989, 1991; Spence et al., 2004). The other source of retina regeneration, which is the current focus of this work, consists of a pool of stem/progenitor cells located in the ciliary region of the chick eye. These cells proliferate and eventually differentiate as long as there is either ectopic FGF or ectopic Sonic hedgehog (Shh) avail-

ABBREVIATIONS CMZ ciliary marginal zone **PE** pigmented epithelium **NPE** nonpigmented epithelium **RPE** retina pigmented epithelium **FGF** fibroblast growth factor **FGFR** fibroblast growth factor receptor **MAPK** mitogen-activated protein kinase **Erk** extracellular signal-regulated kinase **pErk** phosphorylated ERK **MEK** ERK kinase **Shh** Sonic hedgehog **Hh** Hedgehog **Ptc** Patched **PH3** phosphohistone H3

The Supplementary Material referred to in this article can be found at http://www.interscience.wiley.com/jpages/1058-8388/suppmat

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Grant sponsor: National Institutes of Health; Grant number: EY 014197.

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DOI 10.1002/dvdy.21115
Published online 26 March 2007 in Wiley InterScience (www.interscience.wiley.com).

able (Spence et al., 2004). At E4 (time at which retinectomies are performed), the ciliary region is not well defined and contains the putative ciliary body and ciliary marginal zone (CB/CMZ), which is immunopositive for collagen IX, a CB/CMZ marker (Spence et al., 2004). In addition, at E4 most cells in the developing CB/CMZ coexpress Pax6 and Chx10. This coexpression identifies the presence of retinal progenitor cells (Belecky-Adams et al., 1997; Fischer and Reh, 2000; Fischer et al., 2002; Spence et al., 2004). As the eye develops, the CB and the CMZ become well defined so that the CB is composed of a two-layered epithelium, the pigmented epithelium (PE) and nonpigmented epithelium (NPE). The CMZ becomes a transitional zone between the peripheral retina and the CB (Perron et al., 1998; Fischer and Reh, 2000, 2003). Only amphibians, fish, and birds house progenitor cells in the CMZ that are able to proliferate and add new neurons to the retina postnatally (reviewed in Haynes and Del Rio-Tsonis, 2004; Hitchcock et al., 2004). In the chick, this region is only active for the first 3 weeks posthatch (Fischer and Reh, 2000). Early posthatch chicks also house stem cells in the CB that are able to proliferate in response to injection of exogenous growth factors such as insulin, epithelial growth factor, and FGF2 (Fischer and Reh, 2003).

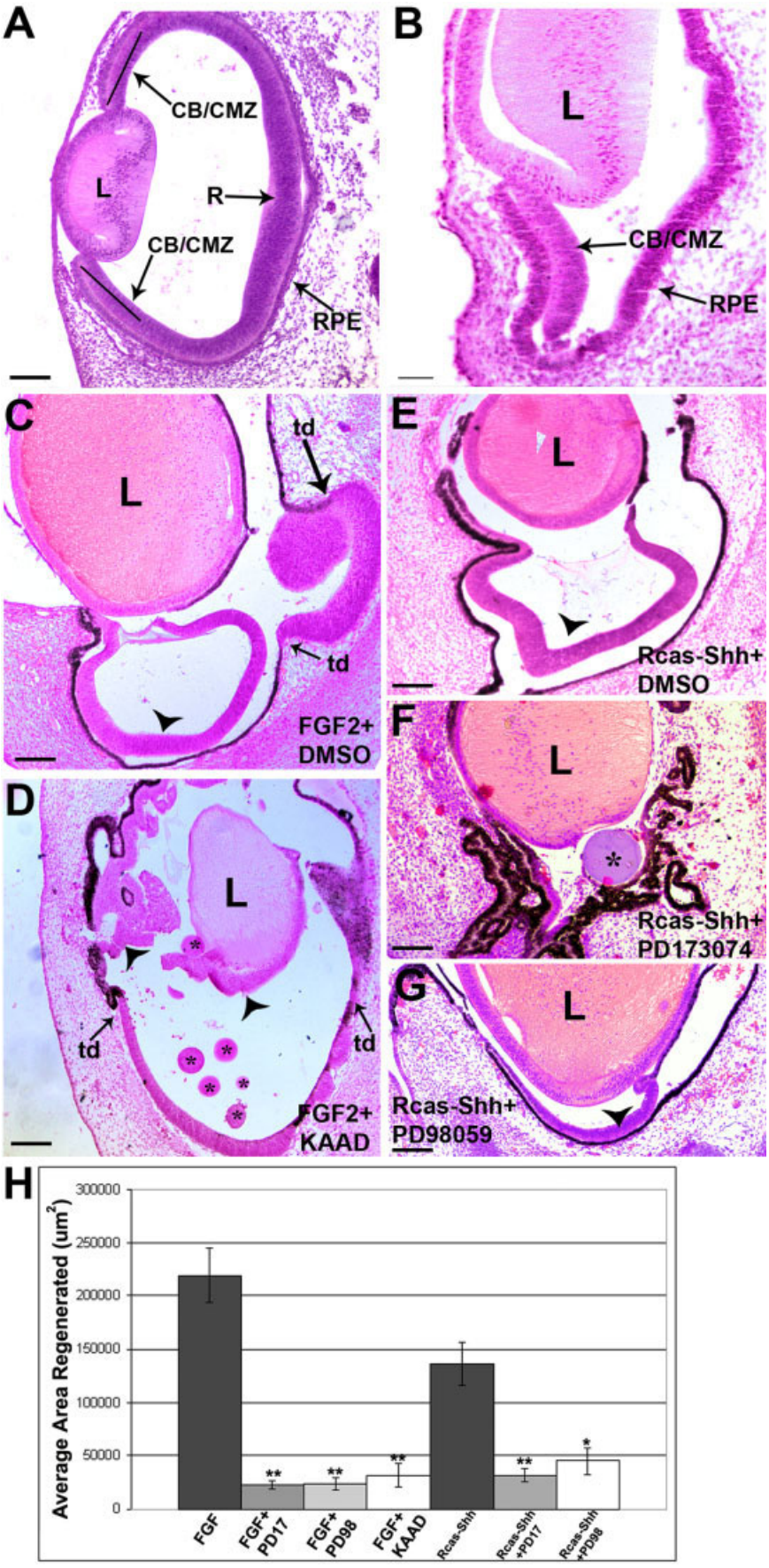
Moshiri et al. (2005) have shown that Shh signaling is important in the CMZ as it is able to stimulate proliferation in the posthatch chick eye. Interfering with the Hedgehog (Hh) pathway inhibits the ability of progenitor cells in this region to proliferate. Similarly, mice with overactive Hh signaling maintain a progenitor population in the CB. In fact, these progenitors are able to give rise to new neurons in an injured retina mouse model (Moshiri and Reh, 2004). In the embryonic chick, isolated cells from the anterior region of E9 eyes retain the ability to proliferate in vitro, and when incubated in rotation culture assays, they form laminar structures containing all nuclear retinal cell types (Willbold and Layer, 1992). Consistent with these reports, we have observed that the chick can regenerate the retina from the CB/CMZ in vivo until at least E5 when stimulated with FGF2. However, the regeneration stimulated by FGF2 at E5 does not appear as robust as the regeneration stimulated by FGF2 at E4 (Spence and Del Rio-Tsonis, unpublished observations). Therefore, it appears that, throughout development, the potential of the stem/progenitor cells in the CB/CMZ to regenerate lost or damaged retina is reduced, but not lost completely. We recently reported that both FGF2 and Shh are able to independently induce retina regeneration from the CB/CMZ in E4 chick eyes (Spence et al., 2004). Thus, both FGF and Shh are important in the regulation of stem/progenitor cells in the CB/CMZ of the embryonic and postnatal chick (Fischer et al., 2002; Fischer and Reh, 2003; Spence et al., 2004; Moshiri et al., 2004, 2005).

Despite the recent data on the role of Shh in regulating progenitor populations and proliferation in embryonic and posthatch chicks and in mice, little is known about the mechanisms by which these cells are regulated. In this study, we used the embryonic chick eye as a model to study how stem/progenitor cells in the CB/CMZ are regulated by FGF2 and Shh, as well as the role of FGF and Hh in maintenance of stem/progenitor cells. We found that basal activity of both pathways is required for retina regeneration to take place, because inhibition of either pathway leads to a reduction of regeneration regardless of the treatment (FGF or Shh). To examine the relationship between the two signaling pathways, we developed an in vitro explant system to identify early signaling events stimulated by FGF2 or Shh. As expected, FGF2 activated a mitogen-activated protein kinase (MAPK) signaling cascade that eventually leads to phosphorylation of extracellular signal-regulated kinase (pErk). Surprisingly, we also found that Shh leads to an increase in pErk and that this phosphorylation event is blocked by the translational inhibitor, cycloheximide. Using pharmacological inhibitors of both the FGF and Hh pathways, we show that Erk phosphorylation is reduced if either pathway is inhibited. In addition, we provide several lines of evidence suggesting that Shh induces pErk by up-regulating FGF and FGF receptor (FGFR) expression, which leads to increased pErk, and ultimately, proliferation of the stem/progenitor cells in the CB/CMZ, leading to regeneration of the retina. Finally, we demonstrate that FGF and Shh are required for cell survival in the CB/CMZ after retina removal, and this trait makes them both interdependent during chick retina regeneration.

RESULTS

Shh and FGF Are Required for Retina Regeneration In Vivo

We previously reported that ectopic FGF2 (also Fig. 1C) or overexpression of Shh (also Fig. 1E) are able to independently induce regeneration from the CB/CMZ of the embryonic chick (Spence et al., 2004). We have also reported that, when regeneration is stimulated with Shh and the FGF pathway is inhibited with the FGFR antagonist PD173074, regeneration is significantly inhibited (also Fig. 1F). To further explore if both of the FGF and Shh pathways are required for regeneration from the CB/CMZ, we stimulated regeneration with FGF2 and simultaneously inhibited the Hh pathway with KAAD (a Hh inhibitor). Compared with FGF2 alone, regeneration from the CB/CMZ was not as robust 3 days after removal of the retina (compare Fig. 1C to D). We quantified regeneration by measuring the total area of retina regenerated from the CB/CMZ using ImagePro (Fig. 1H). Our statistical analysis showed that regeneration stimulated by FGF2 was significantly inhibited by KAAD (compare Fig. 1C with D and H). In addition, as already reported, Shh stimulated regeneration was significantly inhibited by the FGFR antagonist PD173074 (compare Fig. 1E with F and H; Spence et al., 2004). It is well documented that one of the pathways through which FGF2 works is the FGFR/MEK/Erk pathway in different cellular contexts (Galy et al., 2002; Rios-Muñoz et al., 2005). To expand on our findings that PD173074 is able to block Shh stimulated regeneration, we used a MEK inhibitor, PD98059, in combination with retroviral Shh overexpression (Rcas-Shh). Consistent with our FGFR antagonist results, in-



hibition of the downstream effector, MEK, was also able to reduce regeneration (compare Fig. 1E with G and 1H). From this set of experiments, it is clear that the Hh and FGF pathways are codependent. That is, when regeneration is stimulated by one pathway, the other pathway must be functional.

Shh Induces Erk Phosphorylation in the CB/CMZ

To determine whether both the FGF and Hh pathways are working through a common mechanism to induce retina regeneration, we examined early signaling events in the CB/CMZ. To do this, we turned to an in vitro system, using isolated E4 CB/CMZ explants (see the Experimental Procedures section). Since we initially

Fig. 1. Fibroblast growth factor (FGF) and Hedgehog (Hh) signaling are required for retina regeneration in vivo. **A:** A histological section of an embryonic day (E) 4 chick eye at the stage at which retinectomies were performed. **B:** E4 chick eye after the retina has been removed, leaving behind only the ciliary body/ciliary marginal zone (CB/CMZ), lens (L), and retina pigmented epithelium (RPE). Note that the RPE is not heavily pigmented at this stage and has thickened. **C:** E7 chick eye, 3 days after retinectomy and addition of FGF2 plus a control bead soaked in dimethyl sulfoxide (DMSO). In FGF2-treated eyes, regeneration from the CB/CMZ (arrowhead) and transdifferentiation (td) of the RPE can be observed. **D:** Inhibiting the Hh pathway with beads soaked in 200 μM KAAD decreases retina regeneration from the CB/CMZ (arrowheads) when stimulated with FGF2, 3 days after retina removal. Asterisks denote KAAD-soaked beads. Note the arrowhead near the lens points to regeneration from the CB/CMZ that is closely associated with the lens. Transdifferentiating retina is denoted by (td) and arrows. **E:** E7 chick eye, 3 days after retinectomy and addition of Rcas-Shh plus a control bead soaked in DMSO. Only regeneration from the CB/CMZ (arrowhead) can be observed. **F:** Inhibition of FGF receptors with beads soaked in 100 mM PD173074 inhibits regeneration stimulated by Rcas-Shh, 3 days after retina removal. Asterisk denotes a PD173074-soaked bead. **G:** Inhibition of MEK with beads soaked in 100 mM PD98059 decreases Rcas-Shh–stimulated regeneration from the CB/CMZ (arrowhead) 3 days after retina removal. R, retina. **H:** The area of regenerated retinal tissue from all treated eyes was traced and quantified using ImagePro as described in the Experimental Procedures section. These quantitative results support the representative images shown in C–G. Error bars are SEM. $^{*}P < 0.05$, $^{**}P < 0.01$. Scale bars = 100 μm in all panels.

showed that regeneration stimulated by Shh is inhibited using PD173074 (Fig. 1; Spence et al., 2004), we wanted to determine whether Shh is able to activate the MAPK pathway. After placing our explants in culture medium, we added increasing concentrations of FGF2 or Shh and assayed for pErk. We found that, compared with control, both FGF2 (10 μg/ml) and Shh (5 μg/ml and 10 μg/ml) were able to robustly induce Erk phosphorylation after 4 hr of exposure (Fig. 2A). The increase in pErk stimulated by Shh was somewhat surprising, as Shh signaling does not usually act by means of the MAPK pathway. However, several reports support the activation of pErk after stimulating the Hh pathway. In one report, transient overexpression of Gli1 in C3H10T1/2 cells increased the amount of pErk. Additionally, the pErk increase in C3H10T1/2 cells was inhibited with the MEK-inhibitor U0126 (Xie et al., 2001). In another report, Shh was able to stimulate proliferation of rat gastric cells by means of pErk activation, which was also inhibited by an MEK-inhibitor PD98059 (Osawa et al., 2006).

We then carried out tissue explant experiments using 10 μg/ml Shh in combination with PD173074 (FGFR inhibitor), PD98059 (MEK inhibitor), or KAAD. We found that all three inhibitors were able to block pErk stimulated by Shh (Fig. 2B). This finding suggests that Shh leads to activation of the MAPK pathway through FGF receptors, because inhibition of FGFRs blocks pErk stimulated by Shh.

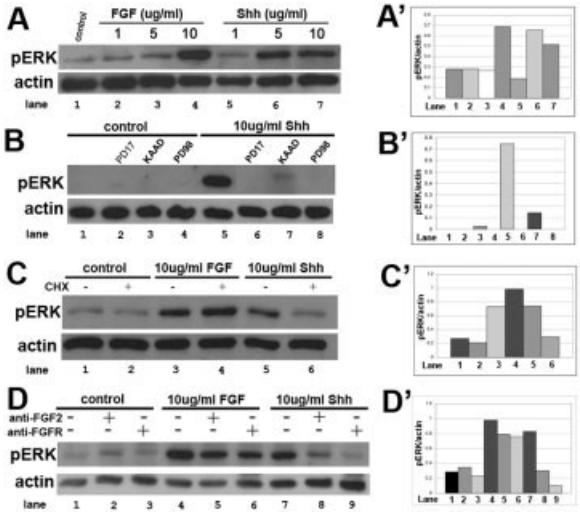


Fig. 2. Early signaling events in the ciliary body/ciliary marginal zone (CB/CMZ) show an intimate connection between fibroblast growth factor (FGF) and Sonic hedgehog (Shh) signaling pathways. **A:** Embryonic day (E) 4 CB/CMZ explants were used as either untreated tissue (control; lane 1) or tissue exposed to 1, 5, or 10 μg/ml of FGF2 (lane 2, 3, 4) or 1, 5, or 10 μg/ml Shh peptide (lane 5, 6, 7). Explants were assayed for phosphorylated extracellular signal-regulated kinase (pErk) using Western blot analysis. Actin was used as a loading control to show that similar amounts of protein were run in each lane. A total of 10 μg/ml of FGF2 and 5 and 10 μg/ml of Shh were able to induce robust Erk phosphorylation. **A′:** Densitometry showing the ratio of pErk/actin in A. **B:** pErk levels were assayed in untreated E4 CB/CMZ explants (control; lane 1) or in E4 explants that were exposed to either FGF receptor (FGFR) inhibitor PD173074 (lane 2), Hh pathway inhibitor KAAD (lane 3) or MEK inhibitor PD98059 (lane 4). Explants were also exposed to 10 μg/ml Shh alone (lane 5) or with PD173074 (lane 6), KAAD (lane 7), or PD98059 (lane 8). Shh was able to induce robust pErk levels, and this finding was abolished by inhibiting FGF/MAPK signaling or Hh signaling. **B′:** Densitometry showing the ratio of pErk/actin in B. **C:** E4 untreated CB/CMZ explants were used as control (lanes 1, 2). E4 explants were treated with 10 μg/ml FGF2 (lanes 3, 4) or 10 μg/ml Shh peptide (lanes 5, 6) for 4 hr. One hour before addition of growth factors, 100 μg/ml of cycloheximide (CHX) was added. CHX did not decrease the amount of pErk in FGF2-treated explants (lane 3 vs. lane 4), but inhibited Erk phosphorylation in Shh treated explants (lane 5 vs. lane 6). **C′:** Densitometry showing the ratio of pErk/actin in C. **D:** E4 untreated CB/CMZ explants were used as control (lanes 1–3). E4 explants were treated with 10 μg/ml FGF2 (lanes 3, 4) or 10 μg/ml Shh (lanes 5, 6) for 4 hr. One hour before addition of growth factors, anti-FGFR or anti-FGF2 was added to the culture medium at a dilution of 1:10. Both anti-FGF2 and anti-FGFR inhibited Erk phosphorylation stimulated by FGF2 (lanes 4–6) as well as Erk phosphorylation stimulated by Shh (lanes 7–9). **D′:** Densitometry showing the ratio of pErk/actin in D.

Shh Stimulated pErk Requires New Protein Synthesis

Shh works by binding its receptor, Patched (Ptc), which releases inhibition on a coreceptor, Smoothened, which then activates one of three Glis (Gli 1, 2, or 3). Gli then moves into the nucleus where it functions as a transcription factor to activate or repress transcription (reviewed in Ruiz i Altaba et al., 2003; Jacob and Briscoe, 2003). Because our data demonstrate that Shh induces pErk in E4 CB/CMZ explants (Fig. 2A), and inhibiting either endogenous Shh or inhibiting the FGF/MAPK pathway in Shh-treated explants inhibits Erk phosphorylation (Fig. 2B), we hypothesized that Shh activates transcription and new protein synthesis, which leads to increased MAPK signaling and increased pErk levels. To test this hypothesis, we cultured CB/CMZ explants with FGF or Shh for 4 hr in the presence or absence of 100 μg/ml cycloheximide (CHX), a protein synthesis inhibitor and examined Erk phosphorylation using Western blot analysis. As expected, pretreatment of tissue with cycloheximide did not reduce pErk levels stimulated by FGF2 because FGF2 binds to FGFRs and consequently elicits the signaling cascade that results in the phosphorylation of Erk (Fig. 2C, lanes 3 and 4). In contrast, cycloheximide treatment was able to block the phosphorylation of Erk in Shh-treated CB/CMZ explants (Fig. 2C lanes 5 and 6). This finding means that Shh needs to elicit new transcription and translation to have a protein product that will initiate a MAPK response. Indeed, pErk levels were similar to basal pErk levels in samples treated with cycloheximide before Shh exposure (compare Fig. 2C lane 6 to lanes 1 and 2). That Shh was able to provoke a protein synthesis-dependent increase in pErk was unexpected as such an intimate relationship between the FGF and Hh pathway has not been well documented. This requirement for new protein synthesis seems to be very rapid. In the chick embryo it has been reported that new proteins are synthesized as early as 2 hr after exposure to developmental toxicants (Pa-

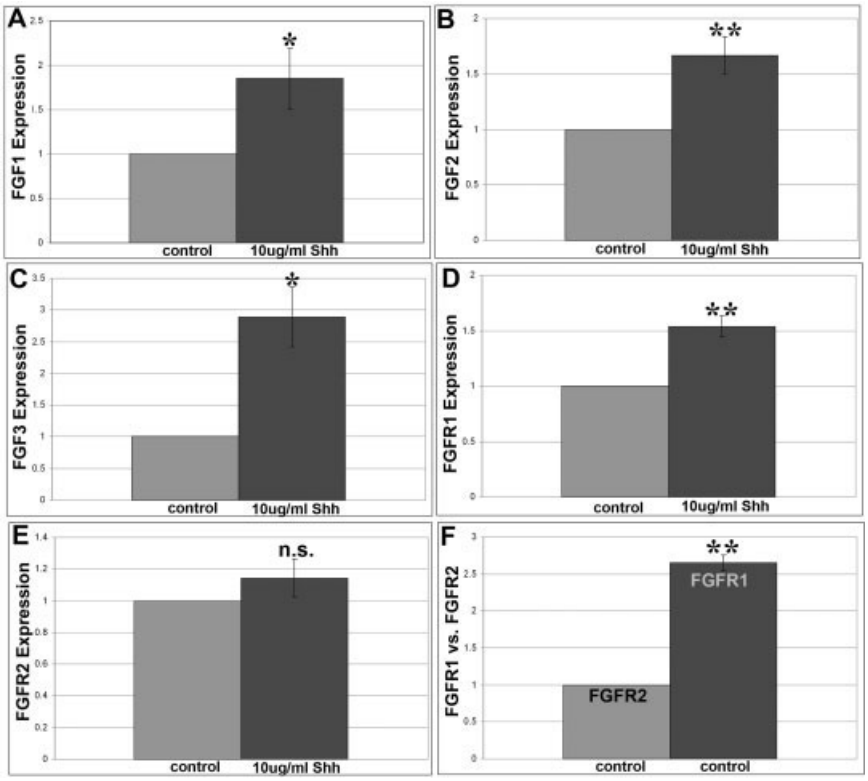


Fig. 3. Sonic hedgehog (Shh) up-regulates fibroblast growth factor (FGF) 1, 2, 3, and FGF receptor-1 (FGFR1). **A:** Quantitative real-time reverse transcriptase-polymerase chain reaction analysis of embryonic day (E) 4 ciliary body/ciliary marginal zone (CB/CMZ) explants stimulated with Shh show that Shh treatment stimulated a significant increase in expression above control tissue for FGF1 (1.85-fold). **B:** Shh triggered a significant increase in FGF2 expression above control tissue (1.65-fold). **C:** Shh caused a significant increase in FGF3 expression above control tissue (2.89-fold). **D:** Shh produced a significant increase in FGFR1 expression above control tissue (1.54-fold). **E:** Shh did not trigger a significant increase in FGFR2 expression above control tissue. **F:** In addition, in control E4 CB/CMZ explants, FGFR1 and FGFR2 levels were compared. FGFR1 was expressed more than 2.5-fold over FGFR2. Error bars are SEM. $*P < 0.05$, $**P < 0.01$. ns = not significant.

paconstantinou et al., 2003) and that Heat Shock proteins reach maximum rates of accumulation after only 5 hr of exposure to transforming growth factor-beta-1 in chicken embryo cells (Takenaka and Hightower, 1993).

Shh Stimulated pErk Is Mediated by FGF Signaling

Because protein synthesis inhibition stops Shh stimulated Erk phosphorylation (Fig. 2C), and because blocking FGFRs using PD173074 inhibits Shh stimulated regeneration after 3 days (Fig. 1) as well reducing levels of pErk stimulated by Shh after 4 hr in our in vitro explant system (Fig. 2B), we hypothesized that Shh may be increasing mRNAs coding for growth factors, or their receptors in the CB/CMZ, which eventually leads to Erk activation. To test this hypothesis, we performed antibody blocking experiments using CB/CMZ explants treated with either FGF2 or Shh. In addition, FGF2 or FGFR blocking antibodies were added to the medium and all treatments were assayed for pErk (Fig. 2D). The FGFR antibody we used for blocking experiments has a high affinity for FGFR1 and a reduced affinity for FGFR2 as described by the manufacturer (Chemicon Antibody MAB125; Venkateswaran et al., 1992). This antibody has also been shown to inhibit FGF activity in chick Müller and retinal cells (Desire et al., 2000). In explants treated with FGF2, the anti-FGF2 or anti-FGFR treatments only slightly reduced pErk levels when compared with FGF2-treated CB/CMZ explants alone (Fig. 2D, lanes 4, 5, 6). This inhibition was not complete and is likely explained by the ratio of FGF2:blocking antibody. Due to the high concentration of antibody used in this experiment (10 μg, a 1:10 dilution), we did not attempt to perform a competition experiment between the blocking antibodies and FGF2. In explants treated with Shh, both anti-FGF2 and anti-FGFR blocked pErk. In fact, both methods of blocking FGF signaling resulted in pErk levels that were similar to basal levels (Fig. 2D, compare lanes 8 and 9 to control lanes 1, 2, and 3). This set of experiments suggests that, in the CB/CMZ, Shh indirectly signals by means of the FGF/FGFR pathway.

Shh Up-regulates *FGF* and *FGFR1* mRNA

To more directly test how Shh may be activating the FGF pathway, we used quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) to determine whether exposure to Shh was able to cause an mRNA increase in any of the members of the FGF pathway. Specifically, we examined *FGF receptors 1* and *2*, because our antibody blocking experiments revealed that blocking FGFR 1 and 2 reduced Shh stimulated pErk (Fig. 2D). We also examined all of the *FGF* ligands that are known in the chick. A list of results can be found in Supplementary Table S1, which can be viewed at http://www.interscience.wiley.com/jpages/1058-8388/suppmat. Here, we focus only on results that showed a significant change in mRNA expression in response to Shh (Fig. 3). We found that adding Shh to CB/CMZ explants significantly increased *FGF1*, *FGF2*, *FGF3*, and *FGFR1* mRNA expression, but not *FGFR2* or other *FGF* ligands (Fig. 3A–E, and Supplementary Table S1). Additionally, as expected, Shh was able to up-regulate *Ptc-1* mRNA in CB/CMZ explants (not shown).

We also observed that, in control CB/CMZ, *FGFR1* was more robustly expressed than *FGFR2* (Fig. 3F). This finding was corroborated with immunohistochemical analysis of FGF receptor expression in the developing eye, where *FGFR1* was abundant in the CB/CMZ, while *FGFR2* was barely detectable in the CB/CMZ (not shown). We were curious to know if the stem/progenitor cells in the CB/CMZ were being regulated specifically

by *FGFR1*, because it is more robustly expressed. To do this, we removed the retina at E4 and added FGF7 (also called keratinocyte growth factor, KGF) to the eye. FGF7 preferentially binds to FGFR2 isoform IIIb (FGFR2-IIIb; reviewed in Itoh and Ornitz, 2004; Mohammadi et al., 2005). After 3 days of exposure to FGF7, we observed some regeneration from the CB/CMZ (not shown), although this was not as robust as regeneration stimulated by FGF2. This regeneration indicates that signaling through multiple FGFRs may stimulate regeneration in the CB/CMZ. At this time, investigation of specific FGFR isoform activity is beyond the scope of this work. That different FGFRs can be involved in inducing retina regeneration is likely correlated with a redundant role for FGFRs, a phenomenon seen in different developmental processes. For example, lung morphogenesis is disturbed only when both FGFR3 and FGFR4 are knocked out during development in mouse, indicating a compensatory developmental mechanism when only one gene is knocked out (Weinstein et al., 1998).

Because Shh was able to regulate components of the FGF signaling pathway, we carried out a similar experiment to determine whether stimulating CB/CMZ explants with FGF2 was able to up-regulate components of the Shh signaling pathway and activate Hh signaling. We chose to examine *Ptc-1* expression, because it is known to be a downstream target of Shh signaling (Spence et al., 2004, and references therein). We found, by real-time RT-PCR, that addition of FGF2 did not change the expression of *Ptc-1* mRNA in CB/CMZ explants (not shown). From these results, we determined that stimulating the CB/CMZ with FGF2 does not activate Hh signaling.

Hh Signaling Is Required for Basal pErk and Erk Phosphorylation Stimulated by FGF

To uncover the effect of the inhibitors used in Figure 1 and on FGF stimulated pErk levels, we added PD173074, PD98059, or KAAD along with 10 μg/ml FGF2 to our explant culture system (Fig. 4). As expected,

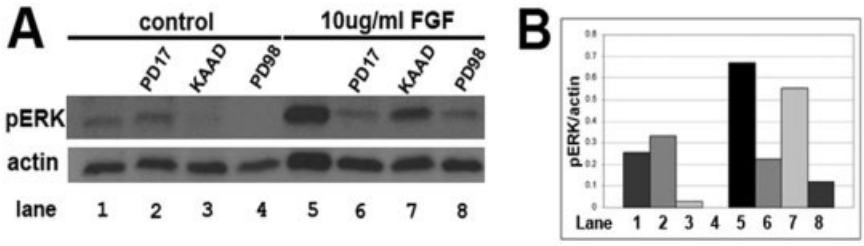


Fig. 4. Hedgehog (Hh) signaling is required for basal phosphorylated extracellular signal-regulated kinase (pErk) as well as fibroblast growth factor (FGF) -induced pErk. **A:** pErk levels were assayed in embryonic day (E) 4 untreated ciliary body/ciliary marginal zone (CB/CMZ) explants (lane 1) or in explants that were exposed to the FGF receptor (FGFR) inhibitor PD173074 (lane 2), the Hh pathway inhibitor KAAD (lane 3), or the MEK inhibitor PD98059 (lane 4). Explants were also exposed to 10 μg/ml FGF2 alone (lane 5) or with PD173074 (lane 6), KAAD (lane 7), or PD98059 (lane 8). Inhibiting the Hh pathway reduced the levels of both basal pErk and FGF-induced pErk. **B:** Densitometry showing the ratio of pErk/actin in A.

FGF induced robust pErk (Fig. 4A, lane 5, and 4B), an effect that was inhibited by PD173074 and PD98059 (Fig. 4A, lanes 6 and 8, and 4B). In addition, the Hh pathway inhibitor KAAD was able to decrease pErk levels in the control (no FGF, KAAD alone, Fig. 4A, lane 3, and 4B). KAAD only reduced pErk levels slightly in the FGF-treated CB/CMZ explants (Fig. 4A, lane 7, and 4B). Therefore, inhibition of the Hh pathway reduced basal levels of pErk but did not considerably change FGF2-stimulated pErk.

Shh and FGF2 Stimulate Proliferation in the CB/CMZ

To further characterize early events stimulated by overexpression of Shh or ectopic FGF2 in the CB/CMZ, we examined cellular events induced by both factors in the CB/CMZ during retina regeneration. Specifically, we examined cell proliferation (Figs. 5, 6) and cell death (Fig. 7). To investigate the outcome of exposing the CB/CMZ to ectopic FGF2, retinectomies were performed on E4 chick eyes and FGF2 was added to optic cups for 24 hr. Heparin beads were used in control experiments. Immunofluoresence labeling was performed on these eyes using an anti-PH3 antibody. The number of PH3-positive cells in the CB/CMZ was recorded (see the Experimental Procedures section) during retina regeneration. A distinction was made between the positive cells present in the nonpigmented ciliary epithelium (NPE) and the pigmented ciliary epithelium (PE) of the CB/CMZ, since we have previously shown that it is the NPE that gives rise to the new retina (Spence et al., 2004). FGF2-treated eyes had significantly more PH3-positive cells in the NPE of the CB/CMZ than controls (FGF2 57.8 ± 4.8 vs. control 41.7 ± 5.4; $P < 0.05$;Fig. 5). To investigate the outcome of exposing the CB/CMZ to ectopic Shh, Rcas-Shh or control Rcas–green fluorescent protein (GFP) virus was injected at E3, the retina was removed at E4, and the eyes were collected 24 hr later. Overexpressing Shh significantly increased the number of PH3-positive cells in the CB/CMZ when compared with control RCAS-GFP infected eyes (Rcas-Shh 64.8 ± 9.7 vs. Rcas-GFP 42.5 ± 4.1; $P < 0.05$; Fig. 5).

Endogenous Hh and FGF Signaling Are Required for Basal Proliferation in the CB/CMZ

Because ectopic Shh or FGF2 is able to increase the number of PH3-positive cells in the CB/CMZ, we hypothesized that inhibiting the Hh pathway using KAAD or inhibiting FGF signaling using PD173074 may reduce the number of PH3-positive cells. To test this hypothesis, we removed the retina from E4 eyes and added dimethyl sulfoxide (DMSO) control beads, 200 μM KAAD beads, or 50 μM PD173074 beads into the optic cup for 24 hr (Fig. 6). Both KAAD- and PD173074-treated eyes had a significant reduction in the number of PH3-positive cells compared with control (Fig. 6; control 41.7 ± 5.4 vs. KAAD 24.8 ± 2.6, $P <$ 0.05; control 41.7 ± 5.4 vs. PD173074

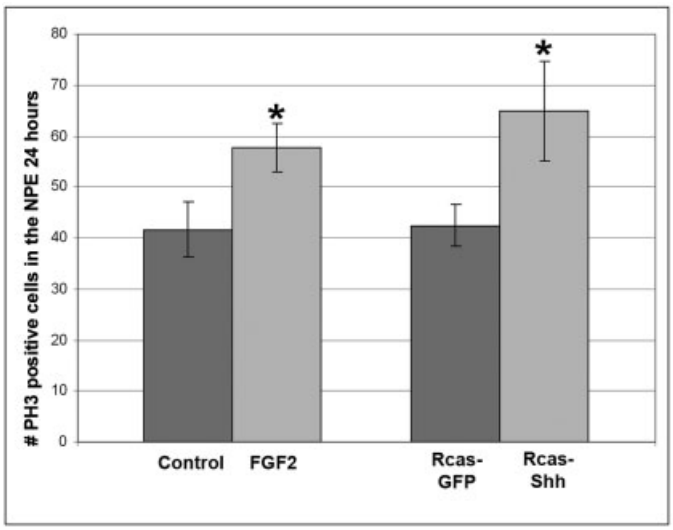


Fig. 5. Increasing levels of fibroblast growth factor-2 (FGF2) or Sonic hedgehog (Shh) increases mitosis. Phosphohistone H3 (PH3) labeling of mitotic cells from embryonic day (E) 4 retinectomized eyes exposed to FGF2 or heparin beads in vivo for 24 hr revealed that FGF2 significantly increased the number mitotic cells in the nonpigmented epithelium (NPE) of the ciliary body/ciliary marginal zone (CB/CMZ; left). Eyes were also injected with Rcas-Shh or Rcas-GFP at E3 and retinas removed at E4. Eyes were collected, sectioned, and assayed for PH3-positive cells 24 hr after retinectomy. Ectopic Shh significantly increased the number of mitotic cells in the NPE of the CB/CMZ (right). *$P < 0.05$, n = 6 sections from three eyes. Heparin beads represent the negative control for FGF treatments and Rcas-GFP the control for Shh treatments. Error bars are SEM.

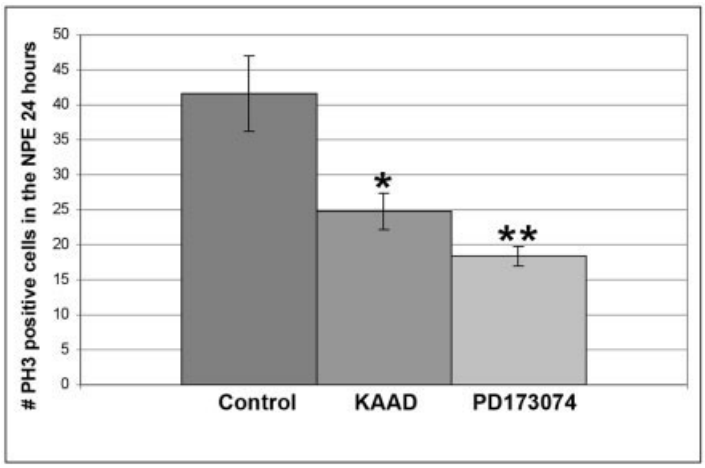


Fig. 6. Hedgehog (Hh) and fibroblast growth factor (FGF) signaling are required for basal proliferation in the nonpigmented epithelium (NPE) of the ciliary body/ciliary marginal zone (CB/CMZ). Retinectomies were performed at embryonic day (E), 4 and chick eyes were exposed to 200 μM KAAD, 50 μM PD173074, or dimethyl sulfoxide (DMSO) for 24 hr. Phosphohistone H3 (PH3) labeling for mitotic cells showed that both KAAD (*$P < 0.05$) and PD173074 (**$P < 0.01$) reduced the number of mitotic cells in the NPE of the CB/CMZ. n = 6 sections from three eyes. Error bars are SEM.

18.33 ± 1.4, $P < 0.01$). Therefore, exposing the CB/CMZ to FGF2 or Shh during retina regeneration increases the number of PH3-positive cells, whereas removing endogenous FGF or Hh signaling using PD173074 and KAAD, respectively, reduces the number of PH3-positive cells.

Hh and FGF Signaling Are Important for Cell Survival in the CB/CMZ

We wanted to examine the amount of cell death that would take place in the NPE of the CB/CMZ when the Hh or FGF pathways are inhibited during retina regeneration. We removed the retina from E4 chick eyes and added DMSO control beads, KAAD-soaked beads (200 μM), or PD173074-soaked beads (50 μM) for 4 hr and assayed for cell death in the NPE of the CB/CMZ using the terminal deoxynucleotidyl transferase–mediated deoxyuridinetriphosphate nick end-labeling (TUNEL) method. We detected a significant increase in cell death when Hh was inhibited in the CB/CMZ compared with DMSO control eyes (control 3.1 ± 0.6 vs. KAAD 17.4 ±2.9; $P < 0.001$; Fig. 7A). We also detected a significant increase in cell death when the CB/CMZ was treated with PD173074, compared with control (control 3.1 ± 0.6 vs. PD173074 7.8 ± 0.9; $P < 0.001$; Fig. 7A).

Because we observed an increase in cell death after inhibiting the FGF or Hh pathway for 4 hr, we hypothesized that the reduction in regeneration after exposure to the inhibitors for each pathway was in part due to the death of many of the cells in the CB/CMZ over time. To examine this possibility, we repeated the above experiment, except we collected the eyes after 24 hr of exposure to KAAD or PD173074. Compared with DMSO control, neither KAAD nor PD173074 induced a significant increase in TUNEL-positive cells (control 123.3 ± 5.9 vs. KAAD 112.5 ± 7.1; control 123.3 ± 5.9 vs. PD173074 91.2 ± 17.6; Fig. 7B).

There was a large increase in the number of TUNEL-positive cells in the untreated eyes from 4 hr (3.1 ± 0.6; Fig. 7A) to 24 hr (123.3 ± 5.9; Fig. 7B). We thought that this large increase in cell death may be due to the absence of the retina and any growth and survival factors that it may provide the CB/CMZ. To determine whether this was the case, we performed retinectomies at E4 and added either DMSO control beads or DMSO control beads plus a piece of the retina into the optic cup and collected the eyes after 24 hr. The number of apoptotic cells in the NPE of the CB/CMZ was recorded during retina regeneration (Fig. 7C). We found that eyes with a piece of retina placed back into the optic cup had significantly fewer TUNEL-positive cells than eyes that received DMSO control beads only (control 123.3 ± 5.9 vs. control + retina 19 ± 7.2; $P < 0.001$). This result indicates that the increase in cell death in

the CB/CMZ between 4 and 24 hr is likely due to the removal of survival factors provided by the retina.

Because we observed an increase in the amount of cell death at 4 hr, but not 24 hr, of treatment with either the Hh or FGF inhibitor, and because the removal of the retina is sufficient to increase the amount of cell death after 24 hr, we believe that the endogenous levels of FGF and Shh appear not to be sufficient to affect survival while they are sufficient to affect the number of proliferating cells. Another possibility could be that other critical retinal factors that contribute to survival are exhausted by 24 hr of retina removal, and under these conditions, changing the levels of endogenous Shh or FGF does not affect the number of apoptotic cells. We further hypothesized that both molecules and their signaling pathways may play a role in cell survival. To determine whether this was the case, after removing the retina from E4 eyes, we added DMSO control, or DMSO control plus FGF2 and collected after 24 hr of exposure. The NPE of the CB/CMZ was assayed for cell death. We found that FGF2 was able to act as a survival factor and significantly reduce the number of cells dying (control 123.3 ± 5.9 vs. FGF2 50.7 ±8.9; $P < 0.001$; Fig. 7C). We repeated the above experiment, except we injected Rcas-GFP or Rcas-Shh at E3. Ectopic Shh was also able to act as a survival factor, significantly reducing the number of apoptotic cells in the NPE of the CB/CMZ during retina regeneration (Rcas-GFP 145 ± 9.1 vs. Rcas-Shh 105.7 ± 12.4; $P < 0.05$; Fig. 7C).

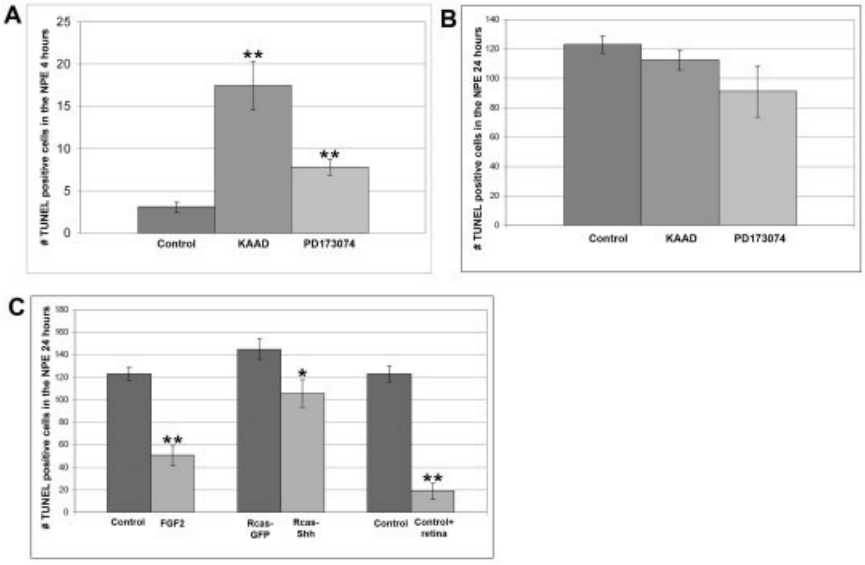


Fig. 7. Fibroblast growth factor (FGF) and Hedgehog (Hh) signaling promote cell survival in the nonpigmented epithelium (NPE) of the ciliary body/ciliary marginal zone (CB/CMZ). **A:** Retinectomies were performed at embryonic day (E) 4, and chick eyes were exposed to 200 μM KAAD, 50 μM PD173074, or dimethyl sulfoxide (DMSO) control beads for 4 hr. Exposure to KAAD (*$P < 0.01$) and PD173074 (**$P < 0.001$) significantly increased the number of terminal deoxynucleotidyl transferase–mediated deoxyuridinetriphosphate nick end-labeling (TUNEL) -positive cells in the NPE of the CB/CMZ. n = 6 sections from three eyes. Error bars are SEM. **B:** Retinectomies were performed at E4, and chick eyes were exposed to 200 μM KAAD, 50 μM PD173074, or DMSO control beads for 24 hr. Exposure to KAAD and PD173074 did not increase the number of TUNEL-positive cells in the NPE of the CB/CMZ. n = 6 sections from three eyes. Error bars are SEM. **C:** Retinectomy was performed on E4 chick eyes. Eyes were then exposed to FGF2 or DMSO control beads (left); Rcas-Shh or Rcas-GFP control (middle); DMSO control beads or DMSO control beads plus a piece of retina (right). Treatment with FGF2 (**$P < 0.001$), Rcas-Shh (*$P < 0.05$), or DMSO control plus retina (**$P < 0.001$) were all able to significantly reduce the number of TUNEL-positive cells compared with their respective controls.

Maintenance of Retinal Progenitor Cell Markers in the CB/CMZ Is Disrupted by Inhibiting Hh Signaling, but Not by Inhibiting the FGF Pathway

It was important to test if manipulating these pathways had any effect on the progenitor cell identity of the CB/CMZ, as it has been previously shown that Hh was required for stem/progenitor cell identity in different tissues (Ericson et al., 1996, 1997; Briscoe et al., 2000; Agius et al., 2004; Xu et al., 2005). To test whether Shh or FGF signaling is required for maintenance of retinal progenitor cell identity in the CB/CMZ, we performed retinectomies at E4, and we assayed DMSO control, KAAD, or PD173074 treated eyes for Pax6/Chx10 coexpression. Cells that coexpress Pax6/Chx10 are considered retinal progenitor cells (Belecky-Adams et al., 1997; Fischer and Reh, 2000; Spence et al., 2004). After 24 hr of exposure to DMSO control beads or 50 μM PD173074-containing beads, we did not find a clear difference in the pattern of Pax6/Chx10 labeling (Fig. 8A,C,D); however, PD173074 caused a significant decrease in the number Pax6-positive cells, but not in the number of Chx10-positive cells (Fig. 8E,F). KAAD-treated eyes on the other hand, showed a significant decrease in Pax6/Chx10 coexpressing cells (Fig. 8B,D). Furthermore, the cells that no longer express a combination of genes have lost Chx10 expression (Fig. 8F).

DISCUSSION

Our experiments help define a complex mechanism by which Shh and FGF signaling interact to induce retina regeneration from the CB/CMZ. In this study, we present data that clearly point to cellular events that are "shared" by both pathways and are critical for retina regeneration.

Models for Shh- and FGF-Stimulated Regeneration

Addition of FGF2 to the optic cup after the retina has been removed leads to increased proliferation (Fig. 5) and induction of retina regeneration from the CB/CMZ (Fig. 1; Spence et al., 2004). Shh overexpression also leads to increased proliferation (Fig. 5), increased FGF signaling (Fig. 2), an increase in expression of FGF family members (Fig. 3), and can induce retina regeneration from the CB/CMZ (Fig. 1; Spence et al., 2004).

There is an obvious model to explain

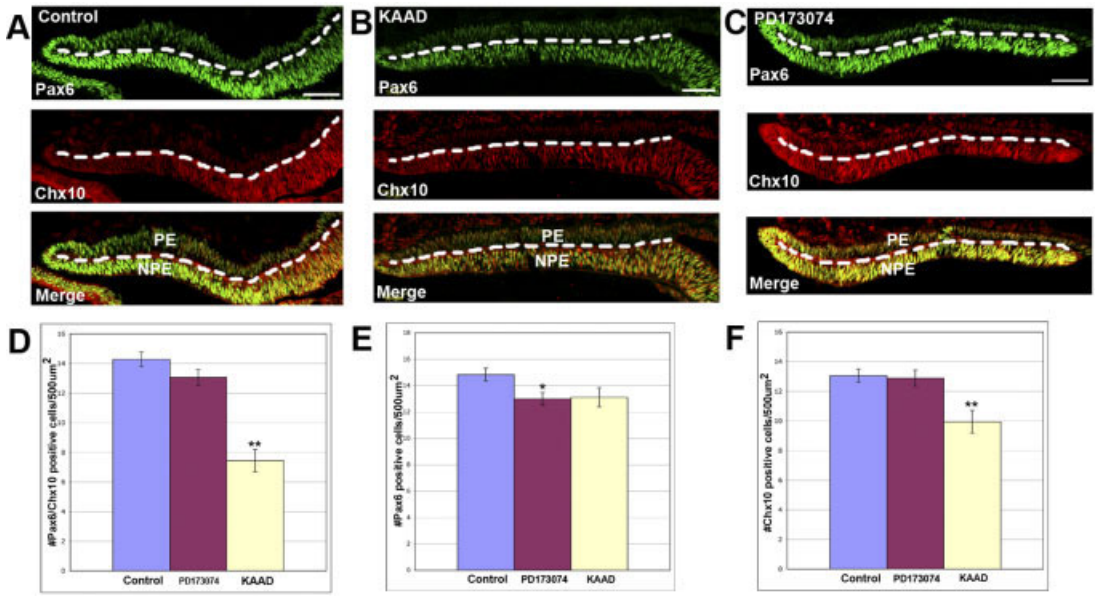


Fig. 8. Maintenance of retinal progenitor cell markers is perturbed by Hedgehog (Hh) inhibition. Retinectomies were performed on embryonic day (E) 4 chick eyes. **A:** Eyes were then exposed to dimethyl sulfoxide (DMSO) for 24 hr. Many cells in the ciliary body/ciliary marginal zone (CB/CMZ) are coexpressing Pax6 and Chx10 as determined by immunohistochemistry. **B:** Operated eyes exposed to KAAD for 24 hr appear to have a difference in Pax6/Chx10 coexpression from control. **C:** Operated eyes exposed to PD173074 for 24 hr show no apparent difference in Pax6/Chx10 coexpression from control. **D:** Quantitation of the area in the nonpigmented epithelium (NPE) containing Pax6/Chx10-positive cells from eyes represented in A–C. Error bars are SEM. $^{**}P < 0.001$. **E:** Quantitation of the area in the NPE containing Pax6-positive cells from eyes represented in A–C. Error bars are SEM. $^{*}P < 0.05$. **F:** Quantitation of the area in the NPE containing Chx10-positive cells from eyes represented in A–C. Error bars are SEM. $^{**}P < 0.01$. Dashed line marks the boundary between the NPE (below the line) and PE (above the line). PE, pigmented ciliary epithelium. Scale bars = 50 μm in all panels.

how Shh overexpression is able to stimulate regeneration, where Shh acts as a regulator of FGF signaling in cells of the CB/CMZ. In this model, Shh overexpression causes an increase in FGF ligands and FGFR1 by means of new transcription as shown in Figure 3, and eventually new protein synthesis. This finding in turn increases the amount of FGF signaling, leading to an increase in pErk (Fig. 2), and the increased levels of pErk lead to and an increase in the number of PH3 positive mitotic cells (Fig. 5).

The codependence of FGF and Hh signaling during retina regeneration from the CB/CMZ could also be explained by the fact that both pathways play a role in survival of the CB/CMZ. Our results suggest that the acute effect of inhibiting either the FGF or Hh pathway in the CB/CMZ after retina removal is to decrease cell survival (Fig. 7). The initial increase in cell death seen by inhibiting either pathway could explain the overall reduction of regeneration over time as well as the decreased pErk activity and reduced number of mitotic cells.

Finally, the CB/CMZ requires endogenous Hh signaling to maintain the identity of its progenitor cells. These progenitors can respond to exogenous growth factors like FGF to proliferate and give rise to new retina. Therefore, FGF and Hh signaling pathways are also interdependent in this case as one pathway is needed to maintain the identity of the progenitor population while the other is required to activate the proliferation of these cells to give rise to new retina.

FGF and Shh Display Proliferative Effects During Retina Regeneration

FGF has been shown to control proliferation of many developing tissues and it is possible that FGF signaling acts upon cyclin genes in this system, just as it does in the developing chick neural tube and during the regulation of cortical precursors (Lobjois et al., 2004; Li and DiCicco-Bloom, 2004). Shh could be controlling proliferation by inducing FGF signaling and subsequently activating cyclin genes indirectly. However, it is also possible that Shh may have an FGF-independent role in regulating cell cycle progression, since it has been demonstrated that Shh is able to regulate key cyclin genes or other important cell cycle regulators, including phosphatases in different contexts (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999; Kenney and Rowitch, 2000; Barnes et al., 2001; Kenney et al., 2003; Oliver et al., 2003; Cayuso et al., 2006; Yu et al., 2006).

Recent evidence from studies in the mouse retina have shown that activation of the Hh pathway acts in a cell-autonomous manner to up-regulate cyclinD1 and increase progenitor cell proliferation (Yu et al., 2006). Activation of Gli3 has also been shown to cause a cell autonomous increase in the number of PH3-positive cells in the developing chick neural tube (Cayuso et al., 2006). Although our studies do not directly address whether Shh overexpression is acting cell autonomously to drive an increase in the number of PH3-positive cells in the CB/CMZ, it is likely that Hh is able to signal in the PH3-positive mitotic cells, because these cells also show immunoreactivity for Gli1 and Gli3, showing that Hh signaling ma-

chinery is present in these cells (Supplementary Figure S1).

Shh Is Able to Up-regulate Members of the FGF Signaling Cascade

As discussed previously, it is possible that one of the ways in which Shh stimulates the CB/CMZ to proliferate and give rise to a regenerated retina is by increasing various members of the FGF signaling pathway, which leads to an increase in FGF signaling activity (Figs. 2, 3). We do not suggest this is the only way in which progenitor cells in the CB/CMZ are regulated, as other molecules have been implicated in regulation of stem/progenitor cells in the CB/CMZ (Fischer and Reh, 2000; Zhao et al., 2002; Kubo et al., 2003, 2005; Liu et al., 2003; Das et al., 2004; Haynes, Gutierrez and Del Rio-Tsonis, unpublished results); however, it is clear from our work that Shh is able to stimulate regeneration in an FGF-dependent manner. Although Shh-mediated regulation of *FGF1*, *2*, *3*, and *FGFR1* has not been previously documented, Shh is able to directly initiate *FGF15* signaling in the diencephalon and midbrain in mice (Saitsu et al., 2005) and can also regulate *FGF19* during forebrain development in zebrafish (Miyake et al., 2005), supporting our observations that Shh induces expression of FGFs in the CB/CMZ of the embryonic chick.

Regeneration Stimulated by FGF2 Requires Basal Levels of Hh Signaling

It is well documented that Hh signaling is required for cell proliferation in many different contexts. For example, Shh is required for cell proliferation of progenitors in the subventricular zone (SVZ) and neocortex in mice (Palma and Ruiz i Altaba, 2004; Palma et al., 2005). In other studies, removal of Shh signaling using conditional null mice for Shh and Smoothened reduced the number of neural progenitors in the SVZ and hippocampus postnatally. In these mice, reduced progenitor cell number was correlated with a marked increase in apoptosis (Machold et al., 2003). Other examples of Hh regulating proliferation and cell death are illustrated in studies on different types of cancer (Qualtrough et al., 2004; Romer et al., 2004; Sanchez et al., 2004; Sanchez and Ruiz i Altaba, 2005). Likewise, Hh signaling is also involved in progenitor cell proliferation and survival during eye development in several different organisms (Stenkamp et al., 2002, and reviewed in Amato et al., 2004, and Moshiri et al., 2004; Wang et al., 2005). Shh has also been implicated in controlling proliferation during regenerative processes. Inhibition of the Hh pathway during lens regeneration in the newt resulted in decreased proliferation and lens fiber differentiation in the lens vesicle (Tsonis et al., 2004). Similarly, treatment of regenerating axolotl tails with the Hh inhibitor cyclopamine significantly reduced blastema cell proliferation, resulting in an incomplete regeneration of the tail (Schnapp et al., 2005). We have shown that Hh signaling has several different biological functions in the CB/CMZ during retina regeneration, consistent with its functions in different contexts and different organisms.

In addition to Shh being able to stimulate retina regeneration from the CB/CMZ, we have shown that FGF2-stimulated regeneration also requires basal Hh signaling (Figs. 1, 4, 8). We have shown that Shh is able to increase the number of mitotic cells as well as act as a survival factor in the CB/CMZ after retina removal (Figs. 5, 7C). Furthermore, removing basal levels of Hh signaling in the CB/CMZ during retina regeneration is sufficient to decrease basal levels of cell proliferation and to induce a wave of cell death soon after retina removal (Figs. 6, 7A; 4 hr). Endogenous Hh signaling is also required for the maintenance of the progenitor cell identity in the CB/CMZ (Fig. 8). Therefore, this combination is likely responsible for the reduced regeneration observed in FGF- stimulated eyes that have also been treated with KAAD (Fig. 1).

Maintenance of Retinal Progenitor Cell Markers in the CB/CMZ

Hh signaling has been shown to be important for progenitor cell maintenance in different cellular contexts. For example, a role for Hh signaling in progenitor cell maintenance has been shown in the developing retina where conditional ablation of Shh in mouse retinas led to a reduction of the retina progenitor (precursor) cell pool (Wang et al., 2005). One of the best examples of Hh signaling specifying and maintaining the identity of neural progenitors takes place during neurogenesis where Shh regulates progenitor cell identity and neuronal fate in the ventral neural tube and developing spinal cord (Ericson et al., 1996, 1997; Briscoe et al., 2000; Agius et al., 2004). In our studies, we observed a significant decrease in Pax6/Chx10-positive cells in eyes that had been treated with KAAD for 24 hr compared with DMSO control-treated eyes (Fig. 8A,B,D). These eyes had a significantly reduced number of Chx10-positive cells (Fig. 8F). On the other hand, inhibition of FGF signaling did not disrupt the retinal progenitor cell population compared with control tissue (Fig. 8C,D). Our results suggest that only Shh regulates the identity of the retinal progenitor population in the CB/CMZ during the process of retina regeneration as defined by the coexpression of Pax6/Chx10.

CONCLUSION

We have demonstrated how both Shh and FGF signaling are interdependent during the process of retina regeneration in the embryonic chick. Although we do not rule out the possibility that other signaling pathways are involved in regulating the CB/CMZ, we propose a model for retina regeneration from the CB/CMZ where FGF and Shh are responsible for stimulating mitosis and proliferation, which leads to regeneration. Shh and FGF are able to induce regeneration from the CB/CMZ by increasing the number of mitotically active cells. The ability of either molecule to do this is dependent on the activity of each other.

In addition, both FGF and Shh signaling pathways are able to act as survival factors in the CB/CMZ. It is clear then, that disrupting FGF or Hh signaling can negatively affect the CB/CMZ by interfering with the ability of either pathway to stimulate proliferation and act as a survival factor.

Finally, endogenous Hh is required to maintain the identity of the retina progenitor population in the CB/CMZ, and any disruption in this pathway will decrease the progenitors available to proliferate and participate in the process of regeneration. Taken together, our results show a complex relationship between FGF and Shh that regulates stem/progenitor cells in the CB/CMZ of the embryonic chick during the process of retina regeneration.

EXPERIMENTAL PROCEDURES

Chick Embryos

Fertilized White Leghorn chicken eggs were purchased from Ohio State University (Columbus, OH) and incubated in a humidified rotating incubator at 38°C.

Preparation of FGF2, KAAD, and Other Pharmacological Agents for In Vivo Studies

Heparin-coated polyacrylamide beads (Sigma) were washed 3 times in 1× phosphate buffered saline. FGF2 (R&D Systems) was resuspended in 1× PBS at a concentration of 1 μg/μl. Heparin beads were then incubated with FGF2 for at least 2 hr before use. To inhibit the Hh pathway we used KAAD, a synthetic form of cyclopamine, which is more potent and not as toxic. A 1 mM KAAD (Toronto Research Chemicals) stock was prepared in 100% ethanol. Affi-gel Blue beads (Bio-Rad) were washed in 1× PBS and dehydrated through a series of ethanol washes of increasing concentration. KAAD stock solution was diluted to 200 μM in DMSO and added to dried beads. Pharmacological inhibitors such as the FGFR inhibitor, PD173074 (Pfizer), and the MEK inhibitor PD98059 (Calbiochem), were resuspended in DMSO at a concentration of 100 mM and incubated in ethanol dehydrated Affi-gel Blue beads. Beads soaked in vehicle (DMSO) alone were used as controls where appropriate.

Retroviral Production, Titration, and Infection

The production of RCAS viruses, as well as their titration and subsequent infection of chick embryos were performed as previously described in Spence et al. (2004). Replication competent Rcas (A) retrovirus engineered to express Shh was a generous gift from Cliff Tabin (Harvard University; Boston, MA). An Rcas construct expressing GFP was a kind gift from Teri Belecky-Adams (IUPUI, Indianapolis, IN) and Ruben Adler (Johns Hopkins University, Baltimore, MD).

Surgical Procedures

A window was made in the egg shell using forceps, and microsurgical removal of the retina was carried out at E4 as previously described (Coulombre and Coulombre, 1965; Park and Hollenberg, 1989, Spence et al., 2004). The entire retina was removed, and the CB/CMZ was left behind, because this region is extremely sticky and difficult to get off. After retina removal, eyes were incubated with either beads or viral vectors as described in Spence et al. (2004).

Tissue Fixation and Sectioning

Tissues processed for histology were fixed in Bouin's fixative for at least 24 hr and embedded in paraffin wax. Transdifferentiated retina is easy to differentiate histologically from retina regenerated from the CB/CMZ as the former lacks RPE and forms a retina with a reverse orientation when compared with a normal developing retina. On the other hand, retina regenerated from the CB/CMZ has normal orientation and it is at least initially associated with the RPE. Tissues used for immunohistochemistry were fixed in 4% formaldehyde, cryoprotected in 30% sucrose, and embedded in O.C.T. freezing medium (Sakura Finetek). All eyes used for histology and immunohistochemistry were sectioned at 10 μm.

Quantitation of Regeneration

To quantitatively measure the amount of retina that regenerated after the different treatments (Fig. 1), we analyzed the area of retina regenerated from three sections of three different eyes (n = 9). Images were obtained using Magnafire image capture software. Captured images were opened in ImagePro, and the regenerating tissue was traced. The area of the trace was determined by ImagePro, and Student's *t*-test was used to assess significance. Error bars in figures represent standard error of the mean (SEM).

In Vitro CB/CMZ Explant Dissection and Tissue Culture

E4 chicks were placed in 1× Hanks' balanced salt solution (HBSS), and the embryonic membranes were removed. The heads were removed and placed in fresh 1× HBSS. For CB/CMZ explants, the cornea was carefully dissected away from the eye and the lens was then removed. Using microscissors, the anterior portion of the eye, corresponding to the presumptive CB/CMZ, was removed and placed in an Eppendorf tube containing 1× HBSS and serum free "Reh's" medium (DMEM/F12 medium, 5 mM Hepes, 0.11% $NaHCO_3$, 0.6% glucose, penicillin 100 units/ml, streptomycin 100 μg/ml; Fuhrmann et al., 2000) at a ratio of 4:1. The tissue was incubated at 37°C.

RNA Isolation of Explants for Real-Time RT-PCR

CB/CMZ explants were incubated for 4 hr as described above, at which time 10 μg/ml FGF or 10 μg/ml Shh-N was added to the culture and the tissue was incubated at 37°C for an additional 4 hr. RNA was then isolated using the Nucleospin II RNA isolation kit (BD Biosciences) following the manufacturer's protocol.

Real-Time RT-PCR (qPCR)

For RT-PCR, RNA was reversed transcribed using ImpromII Reverse Transcriptase (Promega). Real-time PCR was carried out using iQ SYBR Green Master Mix (Bio-Rad) on a RotorGene 3000 Real-Time PCR thermocycler. For PCR primers and annealing temperatures, see Supplementary Table S1. For each sample, the target gene and an internal control were amplified. Quantitation of cDNA for each primer set was determined using the Pfaffl method (Pfaffl, 2001). All exper-

iments described were repeated using at least two separate biological samples. Each biological sample was run in quadruplicate. Significant difference in gene expression between treated tissue and control tissue was determined using the Student's *t*-test, with a sample number of at least six samples (n = 6). Error bars in figures represent SEM.

Immunohistochemistry

Immunohistochemistry was carried out as previously described (Spence et al., 2004).

Western Blotting

Explants were cultured as described above. FGF2 or Shh-N peptide (R&D Systems) was added after the first 4 hr of incubation and cultured for an additional 4 hr. Inhibitors (see Supplementary Table S2: Inhibitors and Antibodies) were added at various time points before FGF2 or Shh was added. The explants were spun down, and the medium removed. The explants were washed with 1× HBSS and resuspended in ice-cold RIPA buffer containing protease inhibitors and phosphatase inhibitors supplied in a RIPA lysis buffer kit (Santa Cruz Biotechnology). Explants were lysed using a sonicator, spun down at 4°C, and the tissue lysate was transferred to a fresh tube on ice. Approximately 15 μg of protein were mixed with 2× sample loading buffer, heated to 95°C for 5 min and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% acrylamide gel. Protein was transferred onto Immobillon-p membrane (Millipore) overnight, and proteins were detected using standard methods. Densitometry was performed using ImageQuant 5.2 software. To determine the relative amount of pErk, Western blot images of pErk and of actin (as a control) were scanned and densitometry comparisons were performed by dividing the density value of pErk by the density value of actin.

TUNEL Assay and Phosphohistone 3

Cell death was assayed using the TUNEL in situ cell death kit (Roche) as previously described (Spence et al., 2004). Phosphorylated histone 3 (PH3) labeling of mitotic cells was carried out using a standard immunohistochemical protocol using an anti-PH3 antibody (Upstate) diluted 1:200.

Cell Counting and Quantitation

Surgeries to remove the retina were performed on E4 embryos. Affigel Blue beads (Bio-Rad) soaked in KAAD or PD173074 solution or DMSO (see Spence et al., 2004) were placed into the optic cups after retinectomy. Embryos were then incubated at 38°C for 4 or 24 hr. Subsequently, eyes were collected, fixed, embedded for frozen sectioning, and sectioned at 12 μm thick. For each treatment, three separate eyes were collected and sectioned. For PH3 and TUNEL analysis, at least two randomly selected sections from each eye were used (total of at least six sections from three eyes). TUNEL assay was performed on sections, and the total number of apoptotic cells in the NPE of the CB/CMZ per section was counted. In the case of the Rcas-GFP and Rcas-Shh experiments, virus was injected into the eye before the retinectomy (as described in the Results section) so the virus had time to infect the tissue and express ectopic GFP or Shh. Retinectomies were performed at E4, and embryos were incubated for different times at 38°C, similar to the KAAD/ PD173074/DMSO experiments. Eyes were processed as described above. Phosphohistone 3 labeling of mitotic cells in Rcas-infected eyes, as well as KAAD/PD173074/DMSO-treated eyes, was done using standard immunohistochemistry. Total number of PH3-positive cells in the NPE of the CB/CMZ per section was counted. Each eye has a dorsal and ventral CB/CMZ, both of which were counted for statistical analysis. For TUNEL and PH3 labeling, statistical analysis was done using the Student's *t*-test. Sample number was at least six, (n = 6). Error bars in figures represent SEM.

Quantitation of Pax6/Chx10-Positive Cells in the CB/CMZ

Eyes that underwent retinectomy at E4 were treated with FGF, KAAD, or DMSO (control). Three different eyes for each treatment were collected, sectioned, and immunostained for Pax6/Chx10. Images of the eyes were then captured on a confocal microscope. Images were imported into Adobe Photoshop, and at least five boxes of 500 μm^2 per section for the three different eyes were placed over the images. The number of cells expressing Pax6, Chx10, or coexpressing Pax6/Chx10 in each box were counted (at least 15 boxes were analyzed per treatment). The Student's *t*-test was used to determine statistical significance. Error bars in figures represent SEM.

ACKNOWLEDGMENTS

We thank Pfizer for providing PD173074 and Dr. Cliff Tabin, Dr. Thomas Jessel, Dr. Ruben Adler, and Dr. Teri Belecky-Adams for providing reagents used in this work. We also thank Dr. Tracy Haynes for assistance with the quantitation data, and Dr. Mike Robinson and M. Natalia Vergara for critical review of this manuscript. J.R.S. was funded by the Foundation Fighting Blindness Canada (FFB-C) student fellowship and K.D.R.T. was funded by NIH grant EY014197.

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