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A novel role of the hedgehog pathway in lens regeneration

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Abstract

Lens regeneration in the adult newt is a classic example of replacing a lost organ by the process of transdifferentiation. After lens removal, the pigmented epithelial cells of the dorsal iris proliferate and dedifferentiate to form a lens vesicle, which subsequently differentiates to form a new lens. In searching for factors that control this remarkable process, we investigated the expression and role of hedgehog pathway members. These molecules are known to affect retina and pigment epithelium morphogenesis and have been recently shown to be involved in repair processes. Here we show that *Shh*, *Ihh*, *ptc-1*, and *ptc-2* are expressed during lens regeneration. The expression of *Shh* and *Ihh* is quite unique since these genes have never been detected in lens. Interestingly, both *Shh* and *Ihh* are only expressed in the regenerating and developing lens, but not in the intact lens. Interfering with the hedgehog pathway results in considerable inhibition of the process of lens regeneration, including decreased cell proliferation as well as interference with lens fiber differentiation in the regenerating lens vesicle. Down-regulation of *ptc-1* was also observed when inhibiting the pathway. These results provide the first evidence of a novel role for the hedgehog pathway in specific regulation of the regenerating lens.
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Keywords: Lens; Transdifferentiation; Regeneration; Shh; Ihh; ptc-1; ptc-2; HIP; KAAD

Introduction

Celebrated as the champion of regeneration research for hundreds of years, the adult newt is the prime paradigm for regenerating entire body parts by transdifferentiation of terminally differentiated cells (Brockes and Kumar, 2002; Tsonis, 2000,2002). Adult newts are able to regenerate their limbs, tail, retina, and lens among other body parts. We have concentrated on lens regeneration because it involves a transformation from one cell type to another and therefore can be regarded as the simplest system for studying regeneration via transdifferentiation. Following lentectomy, a new lens is regenerated by transdifferentiation of the pigmented epithelial cells of the dorsal iris. These cells proliferate as they loose their pigments (dedifferentiation) and eventually differentiate into lens cells. The ventral iris does not contribute to this event, even though it does initially reenter the cell cycle (Del Rio-Tsonis and Tsonis, 2003). Conveniently, the ventral iris can be used as a negative control within the lens regeneration process and can allow for comparisons at the molecular level with the dorsal iris. The restriction implies specific gene expression unique to either the dorsal or the ventral iris. We believe that this restriction must be related to cell signaling, cell–cell communication, and cell interactions since newt ventral irises or cells from both the dorsal and the ventral irises of species unable to regenerate a lens in vivo are able to transdifferentiate in vitro (Eguchi, 1998; Tsonis et al., 2001).

In pursuing experiments along these lines, we decided to examine the expression and role of molecules involved in the hedgehog pathway. Members of the hedgehog (Hh) gene family are key signaling molecules important in many developmental processes in vertebrates. The products of these genes are secreted proteins that act as short- or long-range signals (Drossopoulou et al., 2000; Gritli-Linde et al.,

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2001; Lewis et al., 2001; Panman and Zeller, 2003; Zeng et al., 2001). Sonic hedgehog (Shh) has been found to regulate the dorsoventral patterning of the neural tube and the somites and the anteroposterior axis of the developing limb bud (Drossopoulou et al., 2000; Ekker et al., 1995; Ericson et al., 1996; Panman and Zeller, 2003; Riddle et al., 1993; Schauerte et al., 1998; Wijgerde et al., 2002; Yang et al., 1997). In addition, Shh activity from the ventral forebrain regulates the spatial expression of Pax-6 and therefore plays a crucial role in the development of the midline and consequently of the two eyes (Ekker et al., 1995; Macdonald et al., 1995).

In the developing eye, *Shh* is expressed in the ganglion cell layer of the retina (Perron et al., 2003; Wallace and Raff, 1999; Zhang and Yang, 2001a,b). It has been shown that Shh plays an important role in the differentiation of photoreceptors in the developing eye as well as in controlling the ganglion cell population (Levine et al., 1997; Neumann and Nuesslein-Volhard, 2000; Zhang and Yang, 2001b). Shh signaling from the retinal ganglion cells is also required for normal laminar organization in the vertebrate retina (Wang et al., 2002).

The importance of Shh in eye development has been illustrated by interfering with normal Shh activity. Defective Shh mutants, overexpression of Shh, and inhibition of the hedgehog pathway in several animal models result in eye defects and/or cyclopia (Belloni et al., 1996; Chiang et al., 1996; Huh et al., 1999; Macdonald et al., 1995; Perron et al., 2003; Roessler et al., 1996; Sasagawa et al., 2002; Stenkamp et al., 2000; Zhang and Yang, 2001a). These data suggest that the hedgehog pathway is indeed vital in eye morphogenesis. Overexpression of *Shh* in zebrafish and *Xenopus* embryos reduces the expression of *Pax-6* and affects eye morphogenesis (Macdonald et al., 1995; Perron et al., 2003; Sasagawa et al., 2002). Specifically in *Xenopus*, this overexpression affects the dorsal–ventral and proximodistal axis of the retina (Perron et al., 2003; Sasagawa et al., 2002). In addition, perturbing the hedgehog pathway in embryos that have an eye field established results in severe defects in retinal pigment epithelial cell differentiation (Perron et al., 2003). In chicks, overexpression of Shh causes retina cells to switch fates and become retinal pigment epithelial cells, while inhibition of Shh transforms pigment epithelial cells to neural retina (Zhang and Yang, 2001a).

Indian hedgehog (Ihh) and Desert hedgehog (Dhh) are other members of the hedgehog family that are expressed in the retina. While Ihh has been exclusively detected in the retinal pigmented epithelial cells, Dhh has been found in both the retinal pigmented epithelium (RPE) and the neural retina (Levine et al., 1997; Perron et al., 2003; Takabatake et al., 1997). The functions of hedgehog molecules are mediated by binding to their receptors patched 1 (ptc-1) and patched 2 (ptc-2) (Carpenter et al., 1998; Marigo et al., 1996; Stone et al., 1996), which are also expressed in the eye (Perron et al., 2003; Takabatake et al., 1997; Zhang and Yang, 2001b). Despite the role of the hedgehog pathway in neural retina and RPE development and differentiation and its role in the establishment of the dorsal–ventral and proximodistal axis of the eye, this pathway has not been clearly associated with lens development and differentiation (Levine et al., 1997; Neumann and Nuesslein-Volhard, 2000; Perron et al., 2003; Sasagawa et al., 2002; Zhang and Yang, 2001a,b; Wang et al., 2002). An interesting report, however, has shown that in zebrafish Gli-2 (a downstream effector of the Hh pathway) mutants, the adenohypophysis transdifferentiates to lens (Kondoh et al., 2000). In addition, data have been presented in the literature to indicate that *Shh*, *Ihh*, and their receptors are not expressed in the lens (Levine et al., 1997; Takabatake et al., 1997). However, we have found that *Shh*, *Ihh*, and their receptors are expressed during lens development and regeneration. Our functional studies suggest that the hedgehog pathway is involved in regulating the regenerative process of the lens. These results indicate a novel function of hedgehog members that might bear significance in controlling this unique regenerative process.

Materials and methods

Animals

Adult newts (*Notophthalmus viridescens*) were obtained from Mike Tolley Newt Farm (Nashville, TN). For surgical procedures and euthanasia, the animals were anesthetized using a 0.1% 3-aminobenzoic ethyl ester solution. Eye tissues were collected for histology, in situ hybridization, immunohistochemistry, BrdU staining, and RNA collection. Fixed newt embryos were obtained from Dr. H-G. Simon (Northwestern University, Chicago, IL).

In situ hybridization

In situ hybridizations were carried out as previously described for newt tissues (Del Rio-Tsonis et al., 1999). Probes used were made from newt clones provided by Dr. J. P. Brockes (*Ihh*), Dr. K. Takeshima (*Shh, ptc-1,* and *ptc-2*), and Dr. Y. Imokawa (*Shh*).

Total RNA isolation

Total RNA was extracted from intact dorsal iris, ventral iris, retina, and lens, as well as from these tissues during different stages of regeneration and from developing lenses that were carefully isolated from fixed newt embryos at stages 39–44 according to Khan et al. 1999. Whole iris or whole eyes were also collected. When tissue was abundant, RNA was extracted using TRIzol reagent (Gibco, Grand Island, NY, USA) following the manufacturers instructions. When the amount of tissue was small, RNA isolation was

performed using the NucleoSpin® RNA and Virus Purification Kit (BD Biosciences, Palo Alto, CA). RNA yield was determined by UV spectrophotometry.

Reverse transcription

Up to one microgram of total RNA was used for reverse transcription. For RNA isolated using TRIzol reagent, RNA was incubated with 1unit of RQ1 DNase (Promega, Madison, WI) at 37°C for 20 min. One microliter, 0.5 mM EGTA was added to inactivate the DNase followed by incubation at 65°C for 10 min. The reverse transcriptase steps were performed using a standard protocol using Superscript™ II RNase H-Reverse Transcriptase (Invitrogen, Carlsbad, CA). For reverse transcription of RNA isolated with the NucleoSpin® RNA and Virus Purification Kit, no DNase treatment was included, as it was done during the isolation.

Amplification of cDNA in lens tissue

The Super SMART™ PCR cDNA Synthesis Kit (BD Bioscience) was used to reverse transcribe and amplify 21-day regenerating lens RNA isolated with the NucleoSpin® RNA and Virus Purification Kit following the manufacturer's instructions. PCR was then carried out as described.

PCR primers

PCR primer sequences used include the following: *EF-1α* forward 5′-ATC GAC AAG AGA ACC ATC GA-3′ reverse 5′-GTG ATC ATG TTC TTG ATC AA-3′; *Shh* forward 5′-ACC TCC TCT TTG TAG GCC AGG C-3′ reverse 5′-GTG CCA CTT ACA GAC TTC AGT-3′; *Ihh* forward 5′-GTG CCA CTT ACA GAC TTC AGT-3′reverse 5′-CCA CAG CAA AGC AGG ATA CGA-3′; *ptc-1* forward 5′-AAC AAA AAT TCA ACC AAA CCT C-3′ reverse 5′-TGT CTT CAT TCC AGT TGA TGT G-3′; and *ptc-2* forward 5′-CAC CTC TGT CGA TGG CTT TA-3′ reverse 5′-CAG TTC CTC CTG CCA GTG CA-3′. Resulting PCR product for *EF-2α* is 203 bp, *Shh* is 278 bp, *Ihh* is 198 bp, *ptc-1* is 243 bp, and *ptc-2* is 223 bp.

PCR

PCR reactions were initially incubated at 95°C for 5 min, followed by 35 cycles of 95°C for 45 s, annealing temperature (*EF-1α* 53°C, *Shh* 53°C, *Ihh* 55°C, *ptc-1* 55°C, *ptc-2* 50°C) for 45 s, and extension at 72°C for 1 min. A final extension cycle of 72°C for 5 min was included. *EF-1 α* was used as a positive control, and no RT was added to the negative controls. The PCR product was prestained with an equal volume of a 1:250 dilution of Sybr-green (Molecular Probes, Eugene, OR) for at least 30 min and then separated on a 2% agarose gel and visualized by using the Storm Scanner 1500 (Amersham Biosciences, Piscataway, NJ).

Primary iris culture treated with or without KAAD to determine ptc-1 regulation

Newt irises from 8-day regenerating eyes (irises express *ptc-1* throughout regeneration) were isolated and cultured in vitro with or without KAAD at a concentration of 20 μM. Control samples were treated with the same amount of ethanol present in the KAAD samples. Irises were incubated at room temperature for 24 h and then collected for RNA isolation and processed for examination of *ptc-1* expression.

HIP treatment to determine ptc-1 regulation in iris tissue

Due to differences in culturing conditions (especially temperature), newt irises could not be cultured in vitro with mammalian HIP-expressing cells effectively. Therefore, pellets of HIP-expressing cells or pellets of control GFP-expressing cells were implanted in the newt eye cavity 3 days postlentectomy (see section on inhibition studies using HIP for details on cell pellet preparation). The animals were kept in normal conditions for two more days, and then they were sacrificed for iris collection. RNA was extracted from the iris tissue as described above to examine *ptc-1* expression (as mentioned above, irises express *ptc-1* throughout regeneration; therefore, the timing of this experiment is not critical).

Semiquantitative PCR to determine ptc-1 regulation

To determine relative levels of *ptc-1* mRNA, cDNAs were reverse transcribed from total RNAs isolated from iris tissue incubated with either KAAD or 100% ethanol (see primary iris culture) or from irises dissected from eyes treated with HIP- or GFP-expressing cells as mentioned above. The NucleoSpin® RNA and Virus Purification Kit was used for RNA isolations. To perform a semiquantitative comparison, both *ptc-1* and *EF-1α* were optimized for cycle number, annealing temperature, and cDNA amount (data not shown). *EF-1α* was used as an internal control to normalize *ptc-1* expression levels. Increasing amounts of cDNA were used for both *Ptc-1* and *EF-1α* to demonstrate that values used for quantification were taken from the exponential phase of the PCR and were not obtained from a saturated PCR reaction. PCR was carried out using one cycle of 95°C for 5 min, followed by 34 cycles of 95°C for 45 s, 53°C (*EF-1α*) or 55°C (*ptc-1*) for 45 s, 72°C for 1 min, and a final synthesis cycle at 72°C for 5 min. PCR products were separated on a 2% agarose gel and poststained for 1 h with Sybr-green (Molecular Probes). Images were captured using the Storm Scanner 1500 and quantified using Imagequant software (Amersham Biosciences).

Inhibition studies using KAAD

Heparin beads were incubated in a 200-μM solution of KAAD (a potent cyclopamine derivative: 3-keto, *N*-amino-

ethyl aminocaproyl dihydrocinnamoyl, generous gift from Dr. James Chen and Dr. Philip Beachy) in 100% ethanol for 2 h at 4°C. Control beads were processed in the same way but incubated in 100% ethanol. Fifty-six eyes were lentectomized and KAAD beads were introduced in the eyes. At 5 days postlentectomy, a second set of KAAD beads were placed in the eyes. At the same time, 23 eyes were used as controls following the same procedure but introducing control beads with ethanol into the eyes. The eyes were collected at 20 days postlentectomy and processed for histology. The sections were stained with hematoxylin and eosin.

Inhibition studies using HIP

Mammalian 293 cells were transiently transfected with either a Myc-HIP expression vector or with a control plasmid with GFP (Chuang and McMahon, 1999; Zeng et al., 2001). A hanging drop protocol was followed to pellet the cells. EDTA-treated cells were concentrated to 5×10^7 cells/ml and subsequently aliquoted in 30 μl drops that were placed in a Petri dish, which was inverted and incubated for 3 h at 37°C and 5% CO_2. The same surgical procedure, described for the KAAD experiment, was followed on 18 newt eyes, but this time cell pellets expressing HIP were introduced in the eye cavity instead of beads at 0 and 5 days postlentectomy. Seventeen eyes were used as controls, using cell pellets transfected with the control plasmid. The eyes were collected 20 days postlentectomy and processed for histology as described above.

BrdU experiments and immunohistochemistry

Heparin beads incubated with either KAAD (200 μM) or with 100% ETOH (controls) were introduced into eyes of newts that had been lentectomized 12 days prior. Likewise, HIP- or GFP-expressing cells (controls) were implanted in a parallel set of experiments. In animals used for studying cell proliferation, 1 μl of BrdU solution (10 mM) was then injected into the eye. Twenty-four hours later, the eyes were collected and fixed in 4% formaldehyde solution. The samples were then embedded in OCT (Andwin Scientific, Warner Center, CA) and sectioned at 10 μm. For the β-crystallin expression study, the tissues

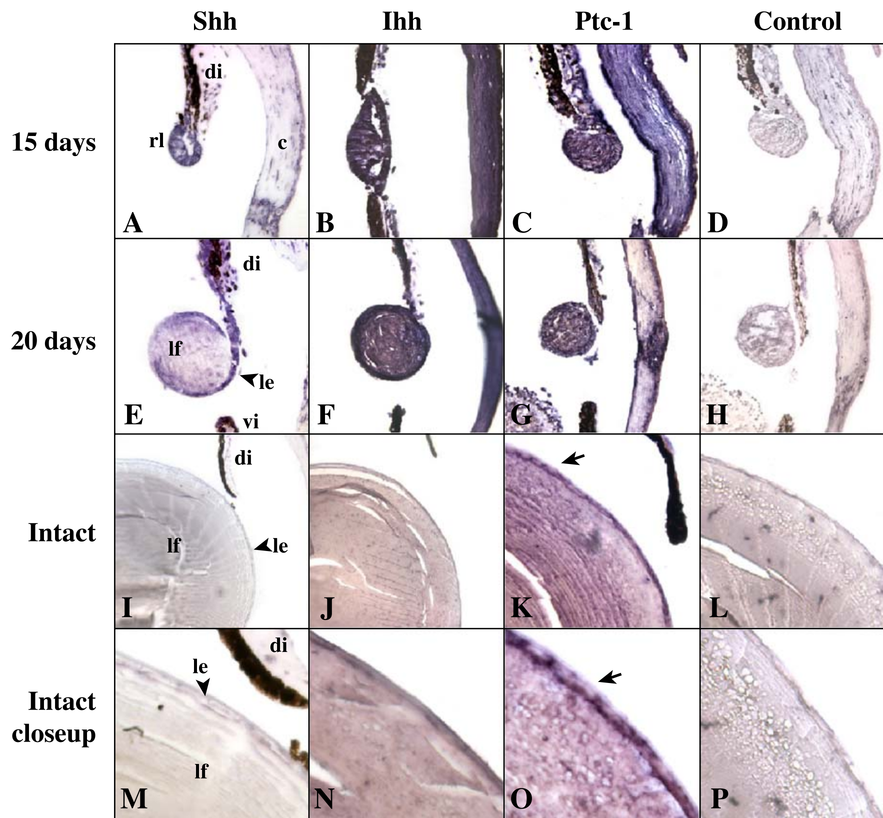


Fig. 1. Expression of *Shh*, *Ihh*, and *ptc-1* in the regenerating lens detected by in situ hybridization. Selected sections from eyes at 15 (A–D), 20 days (E–H) after lentectomy, and of intact eyes (I–P). Note expression of all genes in the regenerating lens vesicle (A–C). As the regenerate developed, the expression of *Ihh* and *Ptc-1* was in both lens epithelium (le) and lens fibers (lf) (F and G), but mostly in the lens epithelium and secondary fibers for *Shh* (E). In the intact lens, only expression of *ptc-1* was detected in the lens epithelium (K and O; arrow). Controls are representative hybridizations with the sense probe for Shh (D, H, L, and P), but all samples had similar background. M–P represent a closeup of the intact lenses (I–L), respectively. Arrowheads in I and M point to the lens epithelium (le). di: Dorsal iris; vi: ventral iris; c: cornea; rl: regenerating lens; le: lens epithelium; lf: lens fibers.

were collected and processed, using the same protocol, 3 days after the beads were implanted. Nine KAAD-treated eyes and eight control eyes were used for the BrdU experiment, as well as six HIP-treated eyes and five GFP control eyes. BrdU was detected using a 1:100 dilution of the primary anti-BrdU antibody (Sigma, St. Louis, MO). To study the expression of β-crystallin, 14 KAAD-treated and 10 control eyes were processed. Ten-micron sections were incubated overnight at room temperature with primary antibody (anti-β-crystallin; designated β6; Sawada et al., 1993) (diluted 1:10 in blocking solution). A 1:10 dilution of FITC conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used to detect the primary antibody. Vectashield (Vector Labs, Burlingame, CA) was used to protect the fluorescence of the samples.

Results and discussion

Lens regeneration in the adult newt begins with proliferation and dedifferentiation of dorsal iris pigment epithelial cells (PECs). Dedifferentiation is the loss of characteristics that define the pigment epithelial cells, such as pigmentation. At about 10 days postlentectomy, a lens vesicle is formed from the depigmented dorsal PECs. Around 12–16 days postlentectomy, the internal layer of the lens vesicle thickens and synthesis of crystallins begins, marking the onset of primary lens fiber differentiation. During days 15–19, proliferation and depigmentation of PECs slows down while primary lens fibers migrate to the center of the lens and nondividing secondary lens fibers appear in the periphery. By 18–20 days, the PECs stop proliferating and the lens fibers continue to accumulate crystallins. Lens regeneration is considered complete by days 25–30 (Del Rio-Tsonis and Tsonis, 2003; Tsonis, 2000).

Shh is specifically turned on during lens regeneration

In our studies, we observed expressions of *Shh*, *Ihh*, and *ptc-1* via in situ hybridization during different stages of lens regeneration. Expression of these genes was evident at the lens vesicle stage and continued throughout all stages of regeneration. In Fig. 1, we show in situ hybridizations using representative stages of lens regeneration. *Shh* and *Ihh* were absent in the intact lens (Figs. 1I, J, M, and N); however, *ptc-1* was expressed in the lens epithelium of the intact lens (Figs. 1K and O). During the process of lens regeneration, all three genes *Shh*, *Ihh*, and *ptc-1* were expressed in the early regenerating lens vesicle (not shown) as well as later stages, including 15 days postlentectomy where the expression patterns were similar (Figs. 1A–D). At a later stage when the lens vesicle has differentiated into distinct layers that include lens fibers and lens epithelium (Figs. 1E–H), the expression seemed more homogenous for *Ihh* and *ptc-1* (Figs. 1F and G, respectively) in the lens epithelium as well as in all lens fibers, whereas *Shh* was mainly expressed in the lens epithelium and secondary lens fibers (Fig. 1E). It is important to note that the conditions for the in situ hybridization studies were optimized for each of the molecules used and hence the differences in background levels and expression levels. Because the iris is heavily pigmented and quite compacted, we were unable to distinguish expression patterns in the iris using in situ hybridization. Also, expression of *ptc-2* was not readily detectable with this method. To corroborate the presence of these genes in the newt eye, the sensitive method of RT-PCR was used. This method was in fact more informative when we examined expression in the iris. We thus examined expression of *Shh*, *Ihh*, *ptc-1*, and *ptc-2* in the following tissues from intact eyes and from eyes undergoing lens regeneration: lens, dorsal iris, ventral iris, and retina. Specific primers for each of the genes were made to avoid possible cross hybridization. *EF-1α* was used as a positive control. The results are presented in Fig. 2. In agreement with the in situ studies, *Shh* and *Ihh* were not found in the intact lens. In addition, *Shh* was not found in the dorsal and ventral irises of the intact eye, while *Ihh* was found in both. However, both receptors *ptc-1* and *ptc-2* were detected in the intact lens and irises. During regeneration, *Shh* and *Ihh* transcripts were found in the regenerating lens and in both dorsal and ventral irises. The fact that activation of *Shh* during regeneration occurs in

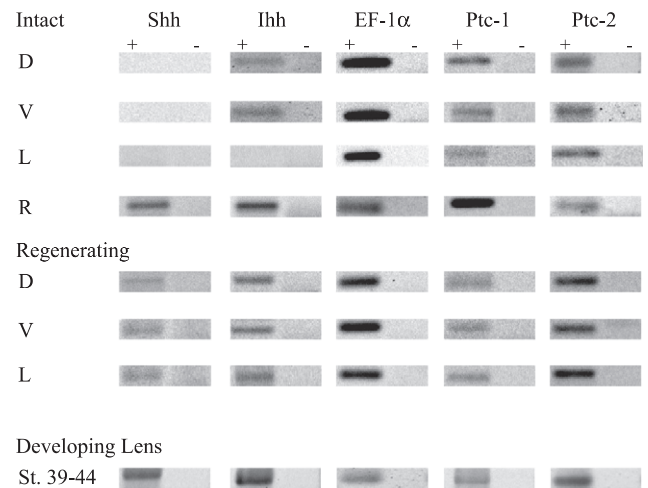


Fig. 2. Expression of *Shh*, *Ihh*, *ptc-1*, and *ptc-2* in tissues of adult (intact and regenerating) and developing newt eyes. Expression studies were performed by RT-PCR. Note that *Shh* and *Ihh* are absent in the intact lens but present in the regenerating lens 21 days postlentectomy. The rest of the panel shows presence or absence of the transcripts in irises and retina of intact and developing eyes (stages 39–44, according to Khan et al., 1999) as well as in eyes undergoing regeneration, representing a range of regeneration stages from 3 to 21 days postlentectomy. *Shh* is the only gene absent in the dorsal and ventral iris of the intact eye. *EF-1* α was used as a control for the RTPCR. + or − indicates the presence or absence of reverse transcriptase (RT).

Table1

Treatment	Affected	Normal
KAAD[a]	16/56 = 28.6%	40/56 = 71.4%
KAAD control	0/23 = 0%	23/23 = 100%
HIP[a]	6/18 = 33.3%[b]	12/18 = 66.7%
HIP control	0/17 = 0%	17/17 = 100%

Regenerating eyes treated with KAAD beads and their respective control beads as well as HIP- and GFP (control)-transfected cells collected 20 days postlentectomy for histological analysis. Affected = vesicles that were 70% or less of the size of a normal regenerating lens, considering differentiation of lens fibers as measure of more mature or larger vesicles.

[a] Indicates that this group shows a statistically significant difference from its corresponding control group ($P < 0.01$) using a chi-square test.

[b] 3/18 had no lens = 17%.

both dorsal and ventral irises deserves some attention, especially as it pertains to cell proliferation (see later section). It is possible that *Shh* expression in the irises is related to the activation of the cell cycle since it is known that both dorsal and ventral irises reenter the cell cycle upon lens removal, even though the rate of proliferation is much higher in the dorsal iris (Reyer, 1977).

We also examined the expression of these genes in the lens of newt embryos (stages 39–44, according to Khan et al., 1999). Interestingly, we found expression for all the genes examined (Fig. 2). Expression of *Shh* and *Ihh* has never been reported in the developing or mature lens in other animals, such as chick or mouse. Therefore, it seems that in newts, these genes might be uniquely expressed during lens formation, get turned off in the mature lens, but can be reactivated during regeneration. In all, our expression studies clearly show that *Shh* is transcriptionally activated in the postlentectomy iris and in the regenerating lens, while *Ihh* is activated in the regenerating lens.

Inhibition of the hedgehog pathway interferes with the process of lens regeneration

Having made this initial observation, we decided to examine the effects of inhibition of the hedgehog pathway on the process of lens regeneration. One of most widely used methods to inhibit the hedgehog pathway is to treat cells or organisms with cyclopamine (Cooper et al., 1998; Incardona et al., 1998; Taipale et al., 2000). This steroidal compound interferes with the downstream factor Smoothened (Chen et al., 2002; Taipale et al., 2000) and is a standard choice in inhibiting the pathway. Another method consists of implanting mammalian cells transiently expressing hedgehog interacting protein (HIP). This protein binds hedgehog molecules and prevents access to their receptors, thus interfering with the downstream signaling targets (Chuang and McMahon, 1999; Zeng et al., 2001).

We used both methods during lens regeneration. HIP-expressing cells or KAAD-soaked beads were implanted into the eye cavity at zero and five days postlentectomy (see Materials and methods). KAAD is a synthetic form of cyclopamine that is 10–20 times more potent and less toxic (Taipale et al., 2000). In control eyes, transfected cells with a control plasmid or ETOH-soaked beads were implanted. Out of the 18 eyes treated with HIP-expressing cells, close to 33% of the eyes were affected showing smaller regenerating lens vesicles (vesicles were considered affected if they were 70% or less in size of a normal regenerating lens, considering as a measure the degree of lens fiber differentiation), including about 17% of the cases with no lens regeneration at all. All 17 eyes treated with control plasmid-expressing cells had normal regenerating lenses (Table 1 and Figs. 3A and B). Of 56 eyes examined in the KAAD experiment, lens morphogenesis was affected in nearly 30% of the cases, even though no cases of complete absence of vesicles were observed (Table 1 and Fig. 3C). This difference between the two treatments is probably attributed to the method of delivery or because these compounds have different modes of action. In addition, KAAD effects seem to be reversible; therefore, the method of delivery is critical. Heparin beads do not allow for a prolonged or slow delivery of the substance being used; thus, we applied KAAD beads at least two

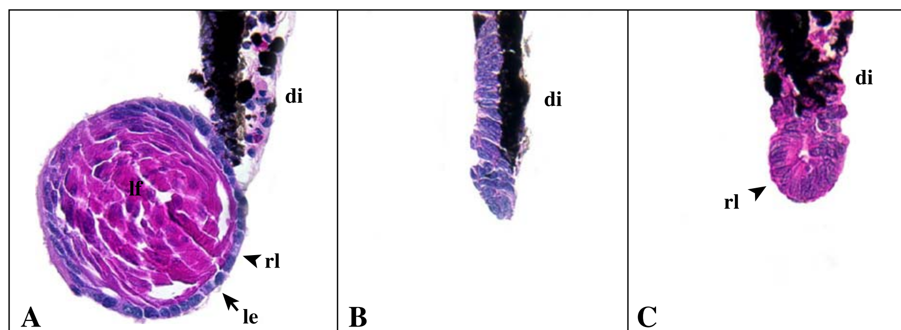
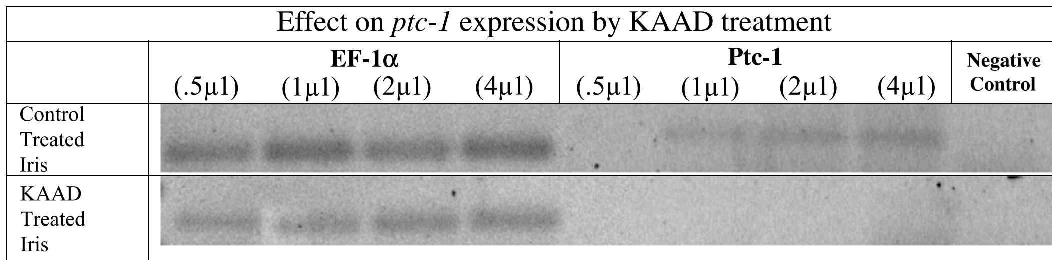
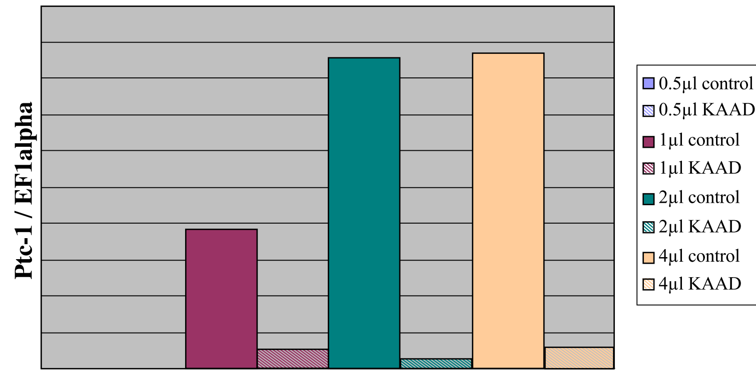


Fig. 3. Representative sections from control, HIP, and KAAD-treated lentectomized eyes showing the effects on the morphology of the regenerating lens. The eyes were collected 20 days postlentectomy and processed for histology; the sections were stained with hematoxylin and eosin. (A) Control regenerated lens, with extensive differentiation of lens fibers (lf). (B) HIP-treated eye showing no lens regeneration from the dorsal iris (di). Only a small depigmented tip is shown, similar to what the ventral iris is capable of at this stage. (C) KAAD-treated eye showing a small lens vesicle without apparent fiber differentiation. di: Dorsal iris; rl: regenerating lens; lf: lens fibers; le: lens epithelium.

A



Relative Ptc-1 mRNA levels in KAAD treated vs. control regenerating lens



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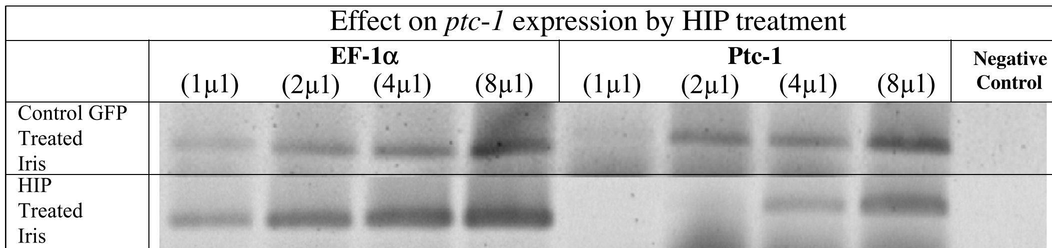

Relative Ptc-1 mRNA levels in HIP treated vs. control regenerating lens

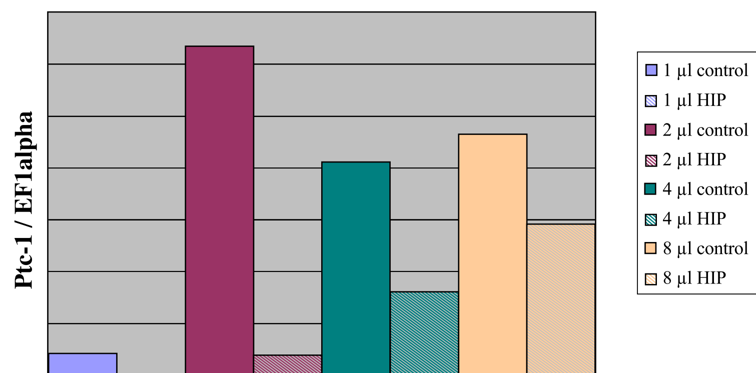

Fig. 4. Semiquantitative RT-PCR showing the effects of KAAD or HIP treatment on *ptc-1* expression. The expression of *ptc-1* in each experiment was determined in relation to *EF-1α* (internal control). (A) Note that treatment of irises undergoing regeneration with KAAD nearly abolished the expression of *ptc-1* (top panel). This was confirmed when quantified using Imagequant software (bottom panel; also see Methods). (B) Similar results were observed on the semiquantitative RT-PCR for the HIP-treated irises, where the expression of *ptc-1* was significantly reduced compared to that of the control experiment (top panel). This was also confirmed using Imagequant software (bottom panel). The negative controls contain no reverse transcriptase. The ratios of *ptc-1/EF-1α* for the lowest doses of the inhibitors in both experimental and control (A) and in experimental (B) were too small to be visible; therefore, scale bars are not presented.

times during the regeneration process. Consequently, considering the issues associated with the methods of delivery, the rate of 30–33% was shown to be significant ($P < 0.01$) using the chi-square test (see Table 1), especially when in the control experiments lens regeneration was normal in 100% of the cases.

However, established molecular tests can corroborate the specificities of these treatments. For example, a commonly used evaluation for cyclopamine effect on the hedgehog pathway is down-regulation of *ptc-1*, which is a downstream target of hedgehog molecules. Therefore, if the KAAD treatment truly affected the pathway, we should observe down-regulation of *ptc-1* in this system. Because our in vivo experiments showed only 30% effect, we developed a controlled and quantitative assay for *ptc-1* regulation. Irises undergoing the process of lens regeneration were isolated 8 days postlentectomy (see Materials and methods) and cultured in vitro for 24 h with a controlled amount of KAAD. Under these optimal conditions, we should be able to tell if KAAD affects *ptc-1* expression. Indeed, when we examined regenerating irises subjected to KAAD treatment and compared for *ptc-1* expression levels with nontreated regenerating irises, *ptc-1* expression was nearly abolished in the treated irises (Fig. 4A). In parallel experiments, we also examined the effects of HIP on regulation of *ptc-1*. As explained in the Materials and methods, for these experiments, cells were implanted into eyes 3 days postlentectomy and irises were collected 48 h later. Inhibition of *ptc-1* expression was shown in these experiments as well (Fig. 4B).

Cell proliferation and lens fiber differentiation are affected if the hedgehog pathway is inhibited during lens regeneration

To test if inhibiting the hedgehog pathway affected cell proliferation during lens regeneration, we treated day 12 regenerating eyes with KAAD and assayed for BrdU incorporation over the next 24 h. This time period was chosen because during normal lens regeneration, cell proliferation in the regenerating vesicle is high (Eguchi and Shingai, 1971). Taking into consideration that the effects of KAAD cannot last over prolonged periods of time (and this can explain the 30% effect seen in our in vivo assays; see discussion above), we decided to treat at this critical time and only for a period of 24 h. Thus, this assay is more likely to accurately measure the effects of KAAD treatments on cell proliferation. The labeled cells in all treated eyes were counted. Indeed, regenerating eyes treated under these conditions showed that cell proliferation in the lens vesicle was significantly affected ($P < 0.001$) (Fig. 5A). Parallel experiments with implantation of HIP-expressing cells showed that proliferation in the lens vesicle was affected with this treatment as well ($P < 0.005$) (Fig. 5B). These results strongly indicate that the hedgehog pathway regulates cell division in this

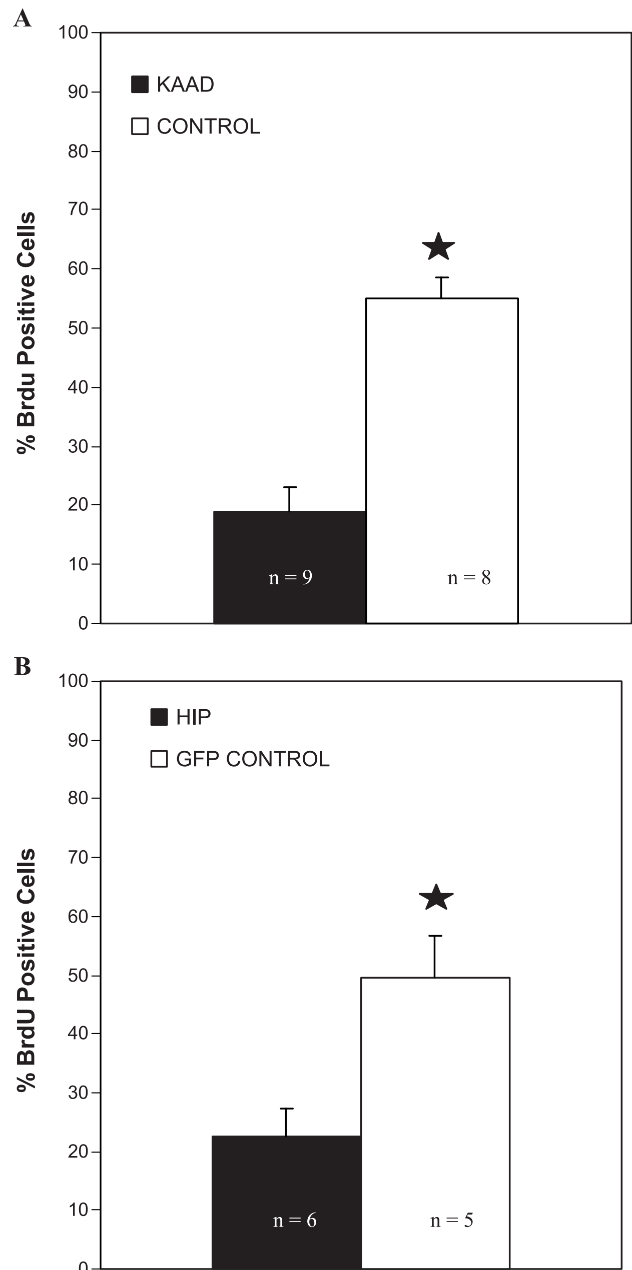

Fig. 5. (A) Effects on cell proliferation in KADD-treated eyes undergoing lens regeneration. Cells in the lens vesicle showed close to 55% BrdU positive cells compared to the KAAD-treated eyes, where the BrdU positive cells dropped to nearly 20%. Error bars are standard error of mean. All cells in the central portion of each lens were counted; control lens had an average of 54 cells but similar regions of KAAD-treated eyes had only 19 cells on an average. *Denotes statistical significance ($P < 0.001$) using Student's *t* test. (B) HIP inhibits cell proliferation in eyes undergoing lens regeneration. Cells of the regenerating lens that were treated with control GFP-expressing cells have approximately 50% of their cells labeled with BrdU, whereas eyes treated with HIP-expressing cells have only 22% of their cells labeled with BrdU. Error bars are standard error of mean. An average of 31 cells per eye per section were counted for HIP and control-treated eyes. *Denotes statistical significance ($P < 0.005$) using Student's *t* test.

process. As it was mentioned above, Shh might be an important early player in the activation of proliferation in both dorsal and ventral iris. Obviously, other factors are additionally involved to restrict regeneration only from the dorsal iris.

In addition, we assayed for lens fiber differentiation by examining for the presence of β-crystallin in regenerating vesicles. Eyes undergoing lens regeneration were treated with KAAD at day 12 and assayed for crystallin expression at day 15. We observed that β-crystallin was not synthesized in eyes that were most affected by KAAD (Figs. 6C and D). Control-regenerating eyes taken at the same stage (day 15 postlentectomy) showed a normal pattern of β-crystallin protein expression, indicating lens fiber differentiation (Figs. 6A and B). We also assayed a 12-day regenerating eye that was not treated with KAAD to compare if at this stage β- crystallin expression had initiated. Indeed at this stage, lens fiber differentiation was evident (Figs. 6E and F). The size of the vesicle at this stage (Fig. 6E) was comparable to the one that had been formed in eyes that were treated with KAAD at 12 days and collected at 15 days postlentectomy (Fig. 6C). The β-crystallin antibody we used is a lens fiber-specific marker (Sawada et al., 1993), suggesting that inhibition of hedgehog proteins affects the differentiation of the regenerating lens fibers as well. We found no evidence (via tunnel assays) that apoptosis increased during KADD treatment (data not shown).

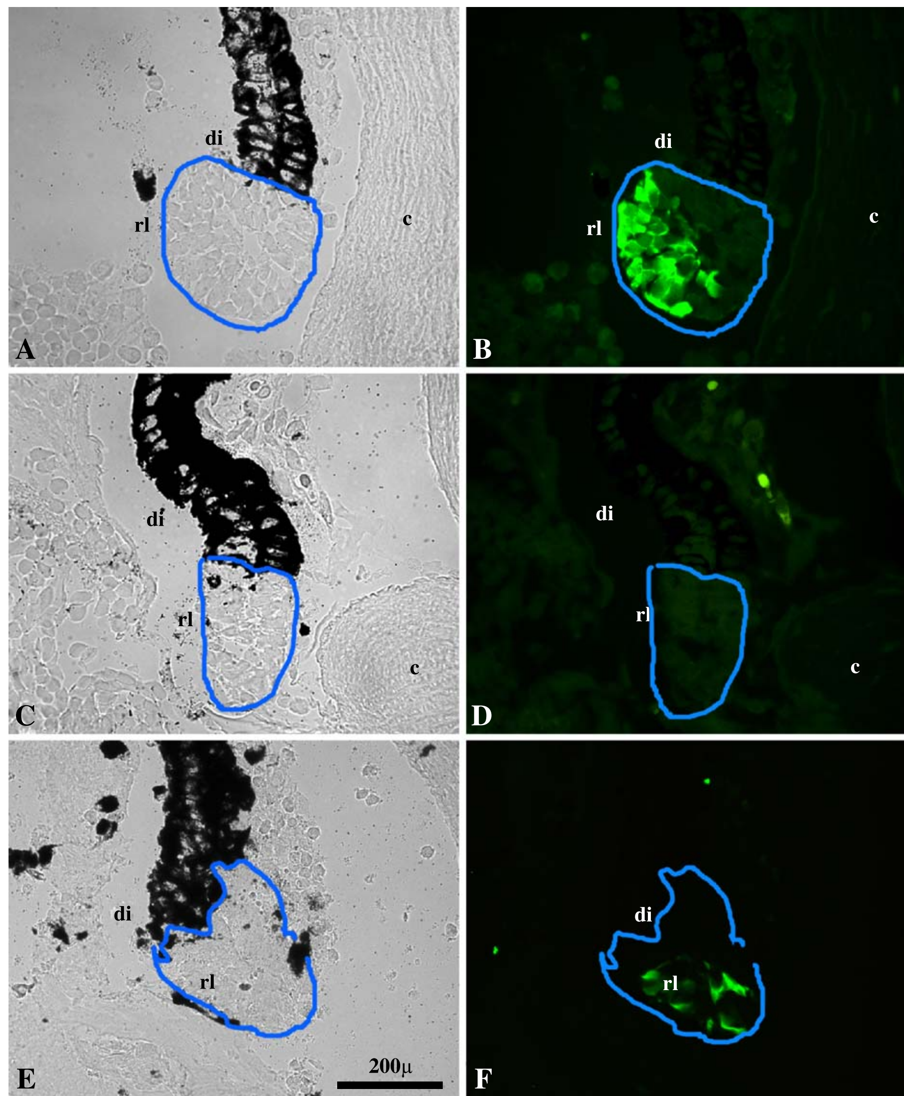

Fig. 6. Crystallin expression in KAAD-treated regenerating eyes. (B) Control-regenerating lens 15 days postlentectomy. Note expression of β-crystallin at the posterior region of the regenerating lens (rl). The cornea (c) is always at the anterior region of the eye. (D) A regenerating lens vesicle from a KAAD-treated eye (15 days postlentectomy) showed no β-crystallin expression. (F) A regenerating control lens vesicle from an untreated eye at 12 days of regeneration showing crystallin expression is included as a comparative control. This lens vesicle was at a similar stage as the one shown in C and D. The blue line outlines the regenerating lens vesicle. (A, C, and E) DIC images of B, D, and F, respectively. di: Dorsal iris; rl: regenerating lens; c: cornea.

In this paper, we provide proof that members of the hedgehog family and their receptors are expressed and involved in the morphogenesis and differentiation of the regenerating lens of the newt. This is the first report to indicate that these genes are expressed in the lens and that they might affect its growth and differentiation. Despite the plethora of data dealing with expression of *Shh*, *Ihh*, and patched in retina and pigment epithelium, expression of these molecules has not been described or focused on during lens development. When the hedgehog pathway is manipulated by overexpressing *Shh* in chick embryos and *Xenopus*, lens morphogenesis is affected. In the first case, the lens is malformed and appears to lack lens fiber differentiation (Zhang and Yang, 2001a), and in the other case, the lens appears smaller than the controls (Sasagawa et al., 2002). Both reports, however, do not elaborate or show any details on the possible effects on lens morphogenesis. Our results strongly suggest that indeed, hedgehog molecules affect lens morphogenesis and that these molecules are recruited for the process of lens regeneration. Other reports support the role of hedgehog molecules in regenerative processes. Studies on limb and fin regeneration have shown that hedgehog molecules are not only expressed during regeneration but are also implicated in the process (Endo et al., 1997; Imokawa and Yoshizato, 1997; Laforest et al., 1998; Quint et al., 2002; Roy and Gardiner, 2002; Roy et al., 2000; Stark et al., 1998; Torok et al., 1999). It is interesting to note here that hedgehog proteins also play a role during tissue repair (Ferguson et al., 1999; Ito et al., 1999; Murakami and Noda, 2000; Vortkamp et al., 1998). Our results indicate that the utilization of the hedgehog pathway is reserved for lens regeneration in newts. The pathway regulates the proliferation and differentiation of the regenerating lens cells. Cell proliferation and differentiation of specific cells types have been shown to be regulated by the hedgehog pathway in other systems (Ingham and McMahon, 2001; Lai et al., 2003; Rowitch et al., 1999; Wetmore, 2003; Yu et al., 2002; Zhang and Yang, 2001a). The novel role of the hedgehog pathway in lens regeneration might bear significance in delineating the mechanisms of such a unique phenomenon.

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