A novel function for Hedgehog signalling in retinal pigment epithelium differentiation

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SUMMARY

Sonic hedgehog is involved in eye field separation along the proximodistal axis. We show that Hh signalling continues to be important in defining aspects of the proximodistal axis as the optic vesicle and optic cup mature. We show that two other Hedgehog proteins, Banded hedgehog and Cephalic hedgehog, related to the mouse Indian hedgehog and Desert hedgehog, respectively, are strongly expressed in the central retinal pigment epithelium but excluded from the peripheral pigment epithelium surrounding the ciliary marginal zone. By contrast, downstream components of the Hedgehog signalling pathway, Gli2, Gli3 and X-Smoothened, are expressed in this narrow peripheral epithelium. We show that this zone contains cells that are in the proliferative state. This equivalent region in the adult mammalian eye, the pigmented ciliary epithelium, has been identified as a zone in which retinal stem cells reside. These data, combined with double labelling and the use of other retinal pigment epithelium markers, show that the retinal pigment epithelium of tadpole embryos has a molecularly distinct peripheral to central axis. In addition, Gli2, Gli3 and X-Smoothened are also expressed in the neural retina, in the most peripheral region of the ciliary marginal zone, where retinal stem cells are found in Xenopus, suggesting that they are good markers for retinal stem cells. To test the role of the Hedgehog pathway at different stages of retinogenesis, we activated the pathway by injecting a dominant-negative form of PKA or blocking it by treating embryos with cyclopamine. Embryos injected or treated at early stages display clear proximodistal defects in the retina. Interestingly, the main phenotype of embryos treated with cyclopamine at late stages is a severe defect in RPE differentiation. This study thus provides new insights into the role of Hedgehog signalling in the formation of the proximodistal axis of the eye and the differentiation of retinal pigment epithelium.

Key words: Retinal pigment epithelium, Retinal stem cells, Hedgehog pathway, Proximodistal axis, Xenopus

INTRODUCTION

In frogs and fish, the retina and the retinal pigment epithelium (RPE) surrounding it grow throughout the life of the animal. The retina grows by adding rings of new neuronal cells at the periphery in a proliferative region containing retinal stem cells called the ciliary marginal zone (CMZ) (Wetts et al., 1989; Dorsky et al., 1995; Perron et al., 1998). Retinoblasts divide in the CMZ and as they are incorporated more centrally, they turn on genes that specify their particular retinal fates (Perron et al., 1998). Recent studies have shown that the most peripheral RPE at the rim of the optic cup has cells that continue to divide postnatally in chick (Fischer and Reh, 2001). This peripheral RPE, with the overlying choroid and underlying non-pigmented epithelium, generates the ciliary body and the iris near the anterior pole of the eye. The mature RPE is a single layer of postmitotic cuboidal cells that play a vital role in ocular metabolism, such as the maintenance of the overlying photoreceptor cells (Boulton and Dayhaw-Barker, 2001). Dysfunction of the RPE, its loss, or separation from the underlying neural retina results in severe photoreceptor degeneration (Jablonski et al., 2000). The RPE also seems to be involved in the maintenance of Müller cells. In conditions in which the RPE is dystrophic because of a genetic mutation, or when it is removed physically, Müller cells undergo degenerative changes (Jablonski et al., 2001). During development, signals from the RPE are involved in retinal histogenesis and layer formation (Layer et al., 1998; Jensen et al., 2001). RPE tissue also has attracted a lot of interest because it can trans-differentiate into a variety of ocular tissues, including retinal neurones (Sakaguchi et al., 1997; Zhao et al., 1997; Reh et al., 1991; Matsuo et al., 1998) (reviewed by Okada, 1980; Reh and Pittack, 1995).

In contrast to these numerous studies on the function of the mature RPE, little is known about its normal development and in particular about the molecular mechanisms involved in
RPE cell differentiation. During the initial stages of vertebrate retinogenesis, cells of the optic vesicle adopt one of two alternate cell fates. Cells in the distalmost part of the vesicle, immediately beneath the surface ectoderm, undergo neural differentiation, while cells in the proximal part differentiate into RPE. This patterning could involve intrinsic cues such as the transcription factors Mitf and Otx2 that are differentially expressed in the prospective neural and RPE domains of the optic vesicle (Mochii et al., 1998; Martinez-Morales et al., 2001). But the patterning of the optic vesicle also depends on interactions with the overlaying surface ectoderm (Hyer et al., 1998). Surface ectoderm-derived FGFs may play an important role in mediating at least part of this interaction by promoting neural fate in the closely apposed neuroepithelium of the distal optic vesicle (Hyer et al., 1998). By contrast, the extra-ocular mesenchyme surrounding the proximal vesicle promotes an RPE fate through an activin-like signal (Fuhrmann et al., 2000). In this study, we present evidence for a role of Hedgehog genes in RPE cell differentiation.

The Hedgehog genes encode secreted signalling proteins that mediate various cell-cell interactions in both vertebrates and invertebrates. In vertebrates, Sonic hedgehog (Shh) is involved in patterning the embryonic limb and spinal cord, and has a role in tooth, lung and hair development (reviewed by Ingham and McMahon, 2001). It has been shown that Shh receptor is composed of at least two proteins: the tumour suppressor protein Patched (Ptc) and the multipass membrane protein Smooveden (Smo) (Murone et al., 1999). The binding between Shh and Ptc is thought to relieve Ptc-mediated inhibition of the activity of Smo (Denef et al., 2000), leading to the activation of transcriptional targets. In Xenopus, two Ptc genes have been identified: Ptc1 and Ptc2 (Takabatake et al., 2000; Koebernick et al., 2001). Three zinc-finger motif transcription factors, Gli1, Gli2 and Gli3, also play critical roles in the mediation and interpretation of Hh signals through the activation and repression of Hh target genes (Ruiz i Altaba, 1999; Koebernick and Pieler, 2002; Ruiz i Altaba et al., 2002). It has been shown that transcriptional targets of the pathway include Ptc1, Ptc2 and Gli1 themselves (Lee et al., 1997; Goodrich et al., 1999; Lewis et al., 1999; Pearse et al., 2001). Several lines of evidences converge to suggest that Shh is involved in early eye development. Targeted gene disruption of Shh in the mouse leads to cyclopia, with no optic stalk, suggesting that Shh is involved in the separation of the eye fields and the formation of the optic stalk (Chiang et al., 1996). In zebrafish, overexpression of shh expands the proximal retina (optic stalk and RPE), at the expense of distal or neural retina (Macdonald et al., 1995; Ekker et al., 1995b). These results suggest that Shh activity, emanating from the rostral midline, is required for the proper formation of the proximodistal axis of the eye. After the eye field separation, a source of Shh emanates from the eye primordium itself in chick (Zhang and Yang, 2001b). This source may play a role in the establishment of the dorsoventral patterning of the eye during the transition from the optic vesicle to the optic cup (Zhang and Yang, 2001b).

Hedgehog genes are expressed in the retina while many cells are undergoing division and differentiation (Wallace and Raff, 1999; Stenkamp et al., 2000; Takabatake et al., 1997). In Drosophila, hh controls retinal development by propagating a wave of photoreceptor differentiation across the eye disc (Greenwood and Struhl, 1999; Dominguez, 1999; Dominguez and Hafen, 1997; Levine et al., 1997). A similar wave of Shh influences neural differentiation in the zebrafish eye (Neumann and Nuesslein-Volhard, 2000). The Shh signal, secreted by early differentiated ganglion cells, has distinct roles at different concentration thresholds. High levels of Shh inhibit rather than promote ganglion cell differentiation in chick retinas. Thus, Shh signals could modulate ganglion cell production and thereby control the progression of the retinal neurogenic wave (Zhang and Yang, 2001a). In vitro data published so far, however, suggest that the roles of shh gene in retinal cell differentiation are very complex. Murine retinal cultures show that Shh can regulate mitogenesis resulting in increased photoreceptor differentiation (Levine et al., 1997) and Müller glia cell differentiation (Jensen and Wallace, 1997). It has been shown in zebrafish that injection of a cocktail of shh antisense oligonucleotides slows or arrests the progression of rod and cone photoreceptor differentiation (Stenkamp et al., 2000). Shh, which is secreted by the axons of ganglion cells also stimulates astrocytes proliferation in the optic nerve (Wallace and Raff, 1999). A role in the retinal organisation has also been suggested since the retina of a mouse carrying a conditional mutation in Shh display extensive laminar disorganisation (Wang et al., 2002).

Other Hedgehog genes are also expressed in vertebrate retina. The zebrafish Tiggywinkle hedgehog (twhh) gene is also expressed in ganglion cells and in the RPE (Neumann and Nuesslein-Volhard, 2000; Stenkamp et al., 2000). Twhh and Shh belong to a same group if phylogenic relationships are taken into account (Ingham and McMahon, 2001). Indian hedgehog (Ihh) has been detected outside the eye, in a layer adjacent to the RPE, and along the optic nerve in the mouse (Wallace and Raff, 1999), but the expression of Desert hedgehog (Dhh) has not been described. We have therefore undertaken a study of the role of all three Hedgehog genes in retinal cell differentiation in Xenopus retina. Three members of the Hedgehog family have previously been isolated in Xenopus, one homologue of Shh (X-shh), one homologue of Ihh, banded hh (X-bhh), and one homologue of Dhh, cephalic hedgehog (X-chh) (Ekker et al., 1995a; Ingham and McMahon, 2001). It has been shown that Hedgehog genes are expressed in the Xenopus adult neural retina (Takabatake et al., 1997) but their expression during Xenopus retinal development is unknown. We therefore first studied the expression of these genes in the developing retina. The expression of Patched (Ptc) genes and Gli1 genes has been studied in the mouse retina (Wallace and Raff, 1999; Wang et al., 2002), but the expression of the other components of the Hedgehog signalling pathway has never been investigated during vertebrate retinogenesis. Therefore, in order to highlight cells that receive Hh signals, we also undertook an analysis of the expression of potential downstream components of the cascade, two patched Xenopus homologues, the homologue of smooveden and three Gli genes, at different stages of retinogenesis. We also investigated the role of Hedgehog genes by activating or blocking the pathway. Interestingly, our experimental approach led us to discover a new role for Hh signalling in RPE cell differentiation. In addition, we found that Hh signalling is important for the proximodistal axis throughout the optic vesicle maturation.
In vitro RNA synthesis and microinjection
Capped dtPKA (Ungar and Moon, 1996) and X-shh (Ekker et al., 1995a) RNAs were prepared from CS2 plasmids after NotI digestion, and pT7TS plasmid after BamHI digestion, respectively, using the mMessage mMachine kit (Ambion). RNAs were injected in a volume of 5 nl at a concentration of 50-100 pg/nl into a single blastomere of embryos at the two-cell stage. Embryos were collected at the tadpole stages, cryostat sectioned and subjected to in situ hybridisation as described below.

Xenopus MITF-A cloning
A partial Xenopus Mitf cDNA was cloned using degenerate PCR primers and stage 21-24 RNA as a template. Conserved regions between human, mouse, chicken and hamster Mitf-1 were used to design degenerate primers: 5DegMi1 ATG GAY CCN GCN YTN CAR ATG; 3DegMi1 TGC GCN CKN GYC TGC ATG; 5DegMi2 ATG GAY CCN GCN YTN CAR ATG; and 3DegMi2 ARD ATN GTN CCY TTR TTC CA. To make cDNA, 1 µg of total RNA and random hexamers were used in a standard 20 µl reaction with M-MLV reverse transcriptase (Promega). PCR amplification of Xenopus Mitf was carried out in a solution containing 2 µl of the cDNA template, 100 ng of the primers, 5DegMi1 and 3DegMi1, and 10% DMSO in the standard PCR solution with AmpliTaq Gold® DNA polymerase (Applied Biosystems). The cycling conditions were as follows: one cycle of 94°C, 9 minutes; five cycles of 94°C for 30 seconds, 37°C for 4 minutes and 72°C for 1 minute; five cycles of 94°C for 30 seconds, 45°C for 4 minutes and 72°C for 1 minute; and 35 cycles of 94°C for 30 seconds, 50°C for 4 minutes and 72°C for 1 minute. A nested PCR reaction was then carried out using 2 µl of the PCR product and degenerate primers, 5DegMi2 and 3DegMi2 in exactly the same solution and conditions as the PCR reaction above. To generate a larger amount of the resulting PCR product, reamplification was carried out using 2 µl of the second PCR product, the same primers and a standard Taq protocol (Roche Applied Science). The clones were ligated into pGEM®-T Easy vector (Promega) and sequenced. The deduced amino acid sequence of three clones was 73% identical to human Mitf (138-247 amino acids; protein reference number I38024). Thus, these cDNAs contained sequence common to heart, neural retina and RPE Mitf clones (Mochii et al., 1998). In order to isolate a sequence unique to retinal pigment epithelial Mitf, or Mitf-A, the Xenopus Mitf sequence data was used to design primers for a 5’ RACE reaction, MixGSP-5′ RACE CTT CGC CTT TCCATA TGG TGT TG and 3Mix262 ATT GTC CTT TTG CCG TTC. 5′ RACE was carried out using stage 35-36 total RNA as template and the SMART RACE cDNA Amplification kit (Clontech). After the initial amplification with MixGSP-5′ RACE and the universal primer mix (UPM) provided in the kit, we used 1.5 µl of this reaction with the nested universal primer (NUP) and 3Mix262 from the kit in a second standard PCR reaction with AmpliTaq Gold. Cycling conditions were 35 cycles for 1 minute each at 94, 58 and 72°C. A single ~750 bp product was obtained, subcloned into pGEM-T-easy and sequenced. The isolated cDNA contained an open reading frame 68% identical to the corresponding region of Human Mitf-A (protein reference number T14752) suggesting that the isolated cDNA is Xenopus Mitf-A.

In situ hybridisation
Digoxigenin (DIG)-labelled antisense RNA probes were generated for Pax2, Pax6, X-bhh, X-shh, X-Ptc-1, X-Ptc-2, X-Smo, Gli1, Gli2, Gli3, Mitf, Xot2, Bren3.0 and Vax2, according to the protocol of the manufacturer (Roche). Whole-mount in situ hybridisation was performed as described previously (Shimamura et al., 1994), with the following change: to visualise expression in the RPE, embryos were bleached (Broadbent and Read, 1999) just before the proteinase K step. After NBT/BCIP (Roche) staining, embryos were vibratome sectioned (50 µm). For double in situ hybridisation, we generated a fluoresceine X-Smo probe according to the protocol of the manufacturer (Roche). X-Smo expression was first revealed with NBT/BCIP, then we inactivated the remaining alkaline phosphatase by incubating the embryos 30 minutes in PBS-EDTA 10 mM at 60°C and we removed the anti-fluorescein antibody bound to the fluorescein-labelled probe by incubating the embryos in Glycine 0.1 M HCl pH2.2 for 10 minutes. Embryos were then washed five times in PBS. X-bhh expression was then revealed with vector red (Vector Laboratories), which can be visualised both in bright field and under fluorescence. Embryos were then vibratome sectioned (50 µm). In situ hybridisation on cryostat sections (12 µm) was performed as previously described (Perron et al., 1998).

BrdU staining
BrdU was injected intra-abdominally, and the animals were allowed to recover for 2-8 hours postinjection. BrdU was detected using the BrdU labelling kit (Roche) after a 45 minute treatment in 2 N HCl. For double staining, the mRNA was first detected by whole-mount in situ hybridisation (as described above). Embryos were then cryostat sectioned and BrdU immunostained.

Cytoplasmic treatment
Cytoplasmic (Toronto Research Chemicals and a gift from William Gaffield) or N-aminooethyl aminoacryl dihydroximomoyl cyclopaamine (KAAD-cyclopaamine; Toronto Research Chemicals) was resuspended in 95% ethanol as previously described (Sukegawa et al., 2000) at a concentration of 5 mM. Embryos were incubated in the dark in 20-100 µM of this cyclopaamine solution diluted in MBS 0.1× (Sive et al., 2000). Control embryos were incubated in MBS 0.1× containing an equivalent dilution of 95% ethanol. These solutions were changed daily.

Immunohistochemistry
Immunohistochemistry was performed on 4% paraformaldehyde fixed tissues. Cryostat sections (12 µm thick) were incubated with primary antibodies (monoclonal anti-RPE antibody XAR1, a gift from Don Sakaguchi; monoclonal anti-rhodopsine R2-12, a gift from N. Colley, monoclonal anti-tubulin, Sigma), and visualised using anti-mouse fluorescent secondary antibodies (Alexa, Molecular Probes).

In vivo lipofection
pCS2-GFP vector (a gift from D. Turner) was transfected into the presumptive region of the retina of stage 18 embryos as previously described (Holt et al., 1990; Dorsky et al., 1995). Embryos were fixed at stage 41 and cryostat sectioned (10 µm). GFP-positive cells were counted and cell types were identified based upon their laminar position and morphology, as previously described (Dorsky et al., 1995).

RESULTS
Xenopus hedgehog genes are expressed in ganglion cells and in the retinal pigment epithelium
We investigated the expression pattern of each of the Hh genes at different stages of retinogenesis, by whole-mount in situ hybridisation with X-shh, X-bhh and X-chh probes (Fig. 1). We then looked at their precise expression patterns in the retina on vibratome sections (Fig. 2). At stage 28, we did not detect any X-shh signal in the optic vesicle (data not shown). We first found a signal for X-shh in the RPE at stage 34 along with faint staining in the ganglion cell layer (Fig. 2A). At stage 42, X-shh expression is strong in the ganglion cell layer (Fig. 2B), but the expression in the RPE is no longer detectable except...
for a faint staining in a cluster of cells in the outer part of the periphery of the retina, possibly newborn photoreceptors and RPE cells (Fig. 2B). Surprisingly, X-bhh is expressed only in the eye of tailbud embryos (Fig. 1C,D). Its expression pattern in the retina is restricted to the RPE from stage 34-35 onwards (Fig. 2C). This expression spreads out in the RPE from the most dorsal to the most ventral part but does not include the most peripheral RPE (Fig. 2C). This progression of expression from the dorsal to the ventral region follows the normal development of the retina – the dorsal part differentiating prior to the ventral part.

We found that expression of X-chh starts to be detected in the RPE slightly later than X-bhh, from stage 35-36 onwards (Fig. 2E). This expression spreads out in the RPE in a very similar way to X-bhh. X-chh is, however, also detected in the hindbrain (Fig. 1E,F). In the retina of stage 42 embryos, the expression of both X-bhh and X-chh is maintained in the RPE but is still completely excluded from the most peripheral RPE, overlaying the CMZ (Fig. 2D,F).

**Xenopus downstream components of the hedgehog pathway are expressed in the retinal pigment epithelium and in retinal stem cells**

To know what cell types in the retina receive the Hh signals produced from RPE cells, we investigated the expression of downstream components of the hedgehog cascade. We therefore performed in situ hybridisation experiments at different stages of the developing retina with the following *Xenopus* probes: X-Patched-1 [X-Ptc-1 (Koebernick et al., 2001; Takabatake et al., 2000)], X-Patched-2 [X-Ptc-2 (Takabatake et al., 2000)], X-Smoothed [X-Smo (Koebernick et al., 2001)], Gli1 (Lee et al., 1997), Gli2 [also called Gli4 (Marine et al., 1997; Ruiz i Altaba, 1998)] and Gli3 (Marine et al., 1997). We found that all these genes are expressed in the developing eye, although more or less strongly depending on the stage of development (Figs 1, 3). For example, in tadpole embryos, X-Ptc-1, X-Ptc-2 and Gli1 expression is strong in the brain but very faint in the retina (Fig. 1I-L,O,P), whereas expression of X-Smo, Gli2 and Gli3 is strong enough in the retina to be detectable in whole embryos (Fig. 1M,N,Q-T). On cross-sections, we found that X-Ptc-1 is faintly expressed in the periphery of the retina at stages 28 and 34, and that its expression decreases with development and only a very faint expression remains in the RPE of stage 42 embryos (Fig. 3A-C). X-Ptc-2 and Gli1 are both expressed in the presumptive RPE and later in the RPE itself (Fig. 3D-I). X-Smo, Gli2 and Gli3 are expressed in the presumptive RPE and in the periphery of the optic vesicle at stage 28 and 34 (Fig. 3J-V). At stage 42, these complex expression patterns become restricted to the most peripheral region of the CMZ containing retinal stem cells, and in the RPE surrounding this region. To confirm that these genes are indeed expressed in the peripheral pigmented epithelium, we performed in situ hybridisation experiments on poorly bleached embryos using a short coloration reaction in order to visualise both the blue staining and the remaining light...
brown pigmentation. We found that these genes are indeed expressed in pigmented cells surrounding the CMZ (Fig. 3R and data not shown). Therefore, some downstream components of the Hh cascade are expressed in the RPE in a pattern complementary to that of X-bhh and X-chh. By comparing expression of X-Smo/Gli2/Gli3 with that of X-bhh/X-chh at stage 42 (Fig. 3L,P, U with Fig. 2D,F), it seems that there is a gap between the hh expression domain and the X-Smo/Gli2/Gli3 expression domain in the RPE. To investigate whether these expression patterns overlap or not during development, we performed double in situ hybridisation experiments on stage 38 embryos with both a X-Smo and a X-bhh probe. In between these two expression domains, cells do not seem to express any of these genes at high levels, suggesting that this might be an intermediate zone (Fig. 4A-C).

**The RPE layer consists of molecular distinct cell populations**

As we have just shown, X-bhh, X-chh and downstream components of the hedgehog cascade are differentially expressed in the RPE of tadpole embryos. These genes may thus represent markers of sub-populations of RPE cells. To study further this molecular subdivision of the RPE, we looked at other RPE markers. XAR1 is a monoclonal antibody, isolated in a large Xenopus marker screen, that marks the RPE (Sakaguchi et al., 1997). We found that XAR1 indeed strongly stains RPE cells from stage 35 onwards. However, it was excluded from the peripheral pigmented epithelium, where X-Smo, Gli2 and Gli3 are expressed (Fig. 4D,E). We then looked at another gene known to be expressed in the RPE, the transcription factor XOtx5 (Vignali et al., 2000; Sauka-Spengler et al., 2001). As previously reported, we found that XOtx5 is expressed in the RPE and the photoreceptor layer (Fig. 4F). We looked at its precise localisation in the RPE and found that it is not expressed in the peripheral pigmented epithelium (Fig. 4F). Together, these results suggest that in addition to Hh cascade genes, other molecules are differentially expressed in the RPE.

It has recently been discovered that some mouse RPE cells can self renew and also generate multipotent neural precursors in vitro, two properties of stem cells (Tropepe et al., 2000). These RPE cells are located in the ciliary margin of the mouse retina, the pigmented ciliary margin. We therefore wondered whether the peripheral pigmented epithelial cells in Xenopus could proliferate in vivo. Long pulses (8 hours) of BrdU incorporation allowed us to show that indeed some of these RPE cells were BrdU positive (Fig. 4G-I). However, no BrdU-
positive cells were detected in the more central RPE where \textit{X-bhh} and \textit{X-chh} are expressed (data not shown). Therefore, only peripheral RPE cells are still proliferating. We then combined in situ hybridisation using \textit{Gli3} as a probe, the expression of which is representative of the Hh signalling pathway, with BrdU staining to ask if the dividing cells express components of the Hh pathway. We found that indeed some BrdU positive cells in the peripheral pigmented epithelium are included in \textit{Gli3} expression domain (Fig. 4J-L). \textit{X-Smo}, \textit{Gli2} and \textit{Gli3} are thus all expressed in a domain containing young and occasionally dividing RPE cells.

\textbf{X-Shh, from the rostral midline, is involved in the establishment of the proximodistal axis of the retina}

It has previously been reported that overexpression of \textit{Shh} or a dominant-negative form of PKA (\textit{dnPKA}) in zebrafish leads to development defects in the eye that suggest involvement in proximodistal patterning (Ungar and Moon, 1996; Ekker et al., 1995a; Macdonald et al., 1995). When we overexpressed \textit{dnPKA} or \textit{shh} in \textit{Xenopus} embryos, we also found such defects (Fig. 5 and data not shown). The ventral region of the retina is transformed in a large optic stalk. This is illustrated by an increased expression of Pax2, an optic stalk marker, and a decreased expression of Pax6, a neural-retina marker (Fig. 5). It is noticeable that the dorsal Pax6 expression remains largely unaffected. Similarly, the morphology of the dorsal neural retina, as well as the dorsal RPE, retains a normal morphology. In \textit{Xenopus}, the dorsal retina is derived from more distal region of the optic cup than the ventral retina. This result thus suggests that Shh signalling in \textit{Xenopus} is also mediated by PKA and may also be involved in the establishment of the proximodistal axis of the retina.

To mimic a loss of Hedgehog function, we took advantage of cyclopamine, a plant steroidal alkaloid that specifically inhibits the cellular response of Hh genes by acting on Smoothened receptor (Cooper et al., 1998; Incardona et al., 1998; Taipale et al., 2000). It has been used in different systems to assess the role of \textit{shh} during development of the pancreas (Kim and Melton, 1998), of the gut (Suкеgawa et al., 2000); in hair follicle morphogenesis (Chiang et al., 1999) and in erythroid differentiation (Detmer et al., 2000). To confirm that the cyclopamine was indeed leading to a loss of Hh function in \textit{Xenopus} embryos, we decided to monitor the expression of genes that are transcriptionally regulated by the Hh pathway in cyclopamine treated embryos. It has been shown that Shh signalling activates \textit{Gli1} (Lee et al., 1997), \textit{Ptc-1} and \textit{Ptc-2} transcription (Goodrich et al., 1999; Pearse et al., 2001; Lewis et al., 1999). We therefore looked at \textit{Gli1}, \textit{X-Ptc-1} and \textit{X-Ptc-2} expression in cyclopamine treated \textit{Xenopus} embryos. We found that \textit{Gli1} expression was the most affected and completely abolished in the retina in early tailbud embryos (Fig. 6A-D). We found that the expression of both Ptc genes was also strongly downregulated in cyclopamine treated embryos (Fig. 6E-H). To

\begin{figure}
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\includegraphics[width=\textwidth]{Fig4.png}
\caption{The retinal pigmented epithelium includes several cell populations. Double in situ hybridisation was performed with \textit{X-Smo} in blue (arrows in A) and \textit{X-bhh} in red (weak in bright field, A; strong under fluorescence, arrows in B). (C) Overlapping staining shows that some cells are negative for expression of both genes in between the two expression domains (white and black arrows give the approximate limits of \textit{X-bhh} and \textit{X-Smo} expression domains, respectively). (D,E) Immunostaining with the antibody XAR1 on retinal cross-section of stage 36 and 42, respectively. Staining is detected in the RPE except in the peripheral pigmented epithelium (bars in E). (F) In situ hybridisation on stages 38 embryos with a \textit{Xotx5} probe. \textit{Xotx5} is expressed in the photoreceptor layer (arrowhead) and strongly in the RPE (arrows), excluding the peripheral pigmented epithelium (bars in E). (G) Staining for BrdU uptake in retina cross-sections of stage 42 embryos. (H) BrdU immunostaining. (I) An overlap of G and H shows that some BrdU-positive cells are present in the peripheral pigmented epithelium (arrowheads). Arrows indicate some BrdU-positive cells in the CMZ. (J-L) Double staining for BrdU uptake and \textit{Gli3} expression. \textit{Gli3} staining is shown in J, BrdU immunostaining in K. Double staining in L shows that some BrdU-positive cells in the peripheral pigmented epithelium are also \textit{Gli3} positive (arrow). le, lens; CMZ, ciliary marginal zone. Scale bar: 45 \textmu m in A-F; 15 \textmu m in G-L.}
\end{figure}
rule out a possible toxic effect, we investigated the expression patterns of Gli2 and Gli3, which have previously been shown not to be transcriptionally regulated by the pathway. Both genes were not affected by the cyclopamine treatment (Fig. 6I-L). These results imply that cyclopamine indeed specifically blocks all the Hh pathways in Xenopus.

To investigate the effect of blocking the Hh cascade on retinal development, we incubated Xenopus embryos in cyclopamine from the two-cell stage. We then analysed the resulting phenotype in tailbud embryos. We found major defects in the ventral part of the retina evocative of a mild cyclopic phenotype. Indeed, the ventral pigment epithelium was not properly closed but was pointing in the direction of the midline as if the eyes had not separated properly (Fig. 7A,B). In addition, some ectopic pigment was present in the middle of the neural retina (Fig. 7A,B). The strength of this phenotype was proportional to the concentration of cyclopamine, 20 μM produced a mild ventral defect, while 100 μM produced a more severe defect shown in Fig. 7B.

To analyse this phenotype further, we performed in situ hybridisation using several markers. As we found that overexpressing X-Shh leads to ectopic expression of Pax2 and to a decrease of Pax6 expression (Fig. 5), we analysed Pax2 and Pax6 expression in these cyclopamine-treated embryos. We found the opposite phenotype, which is an absence of Pax2 expression in the retina and ectopic expression of Pax6 in the optic stalk region (Fig. 7C-F). This result confirms a role for the Hh pathway in the establishment of the proximodistal axis of the retina of Xenopus.

Using an antibody directed against rod photoreceptors, R2-12, or a ganglion cell marker, Brn3.0 (Hirsch and Harris, 1997), we found ectopic photoreceptor layer following the ectopic pigment, and found ectopic ganglion cells in the optic stalk.
region (Fig. 7G-J). We found the same phenotype when we used a modified cyclopamine, the KAAD-cyclopamine, which has been shown to be less toxic than the regular cyclopamine and 10 times more efficient in blocking the Hh pathway (Taipale et al., 2000). Indeed, we found that the strength of the retina phenotype obtained with 20 μM of KAAD-cyclopamine was similar to that obtained with 100 μM of the regular cyclopamine (data not shown). In order to know whether the severe defects found in the ventral region could also result from a dorsoventral defect, we analysed the expression of a marker of the ventral part of the Xenopus retina, Vax2 (Barbieri et al., 1999; Liu et al., 2001) (Fig. 7K,L). Although the ventral part of the retina is morphologically severely affected, Vax2 expression was still present in the ventral half of the retina. Together, these results suggest that the severe phenotype observed in the ventral retina of cyclopamine-treated embryos results from a proximodistal defect rather than from abnormal dorsoventral patterning.

Hedgehog signalling in the retina is involved in RPE cell differentiation

As we were most interested in later roles for the Hh pathways, we decided to incubate Xenopus embryos in cyclopamine solution only from the late neurula stage after the eye fields have clearly separated (stage 20). When we analysed the phenotype of these embryos at stage 40, we indeed did not see cyclopic embryos. However, we observed two obvious major developmental defects. The spinal cord was not as straight as in control embryos, and the pigmentation was abnormal, notably in the RPE (Fig. 8A,B). Indeed, pigmentation around the lens (the peripheral pigmented epithelium) was completely missing, and the remaining pigmentation was less dark than in control embryos.

Using different markers, we then investigated whether the cyclopamine treatment at this stage leads to any retinal defects. Immunostaining using a β-tubulin antibody shows that retinal layers are not affected in these embryos (Fig. 8C,D). Previous experiments have led to the proposal that shh gene may play a role in propagating photoreceptor differentiation across the developing eye of the zebrafish (Stenkamp et al., 2000). However, we detected no difference between control embryos and cyclopamine treated embryos when we analysed photoreceptor differentiation using a rhodopsine antibody (Fig. 8E,F). As other experiments in chick and zebrafish suggest that shh is required to control ganglion cell production (Neumann and Nüsslein-Volhard, 2000; Zhang and Yang, 2001a), we analysed whether the ganglion cell layer forms normally in cyclopamine treated embryos, using Brn3.0 as a marker (Hirsch and Harris, 1997). We did not detect any difference in Brn3.0 expression between control and cyclopamine treated embryos (Fig. 8G,H). To determine whether the inner nuclear layer was affected, we used Xotx2 as a marker. This gene has been shown to be expressed in the CMZ and in the inner nuclear layer in the Xenopus retina of stage 40 (Perron et al., 1998). Again, we did not find any difference in expression of this gene between cyclopamine and control embryos (Fig. 8I,J). In order to quantify the results, we lipofected a GFP construct in neurula embryos that were subsequently incubated in cyclopamine solution. We then counted transfected cells in the mature retina. We found no statistical differences when we compared the percentages of retinal cell types of cyclopamine-treated embryos with those of control embryos (data not shown). All these results suggest that all retinal cell types can differentiate without Hh signalling. Because we occasionally found that the ventral retina was slightly reduced in size and as others have suggested a role for Shh in dorsoventral patterning of the retina (Zhang and Yang, 2001b), we analysed the expression of the ventral marker of the retina, Vax2. We did not see any differences in Vax2 expression between the control and embryos incubated in cyclopamine (Fig. 8K,L). Defects in the ventral retina could result from a problem in the proximodistal patterning of the retina. To test this hypothesis, we looked at Pax6 expression, which is strongly reduced when compared with control embryos (Fig. 8M,N), suggesting that although a cyclops-like phenotype was not detectable, the proximodistal axis was nevertheless affected.
We then further investigated the effect of cyclopamine on RPE differentiation using the antibody XAR1 as a RPE marker. We found that the staining was strongly reduced or even completely absent in the RPE of cyclopamine-treated embryos (Fig. 8O,P). When embryos were incubated in cyclopamine from the two-cell stage, the same absence of XAR1 staining was detected (data not shown). Although the strength of this phenotype was dose dependent, the XAR1 staining was already severely reduced in embryos incubated in 20 $\mu$M of cyclopamine. This result suggests that the Hh pathway is involved in RPE differentiation. To investigate this further, we performed in situ hybridisation on cyclopamine-treated embryos with several other RPE markers. We first wanted to look at an early marker of RPE cell differentiation, such as Mitf (Mochii et al., 1998). We therefore cloned by RT-PCR the Xenopus homologue of Mitf (see Materials and Methods). In wild-type embryos, Mitf is expressed in the developing RPE (Fig. 8Q). We found that the expression of Mitf was decreased in cyclopamine-treated embryos. This effect was the most dramatic in the periphery of the retina where no expression at all was detected, although some expression was visible in the central differentiating RPE (Fig. 8R). We then looked at the expression of other RPE markers, including Xotx5, X-bhh and X-chh. Xotx5 expression is mostly affected in the ventral part of the cyclopamine-treated retina in the RPE layer while its expression in the photoreceptor layer seems normal (Fig. 8S,T). The intensity of the expression of both X-bhh and X-chh is reduced in cyclopamine embryos. This is more striking in the periphery of the retina than in the central RPE, as if the effect follows a gradient of severity from the periphery to the centre (Fig. 8U-X). As a conclusion, although the expression of the various RPE markers we used were differentially affected by the cyclopamine treatment, all were reduced, strongly suggesting that Hh signalling is essential for the proper RPE differentiation.

DISCUSSION

We have shown that the three Xenopus Hh genes are differentially expressed in the retina during development. From the expression of these genes along with several other components of the Hh pathway and other RPE markers, we have highlighted different zones in the RPE. In addition to an early role for X-shh, from the midline to pattern the proximodistal axis of the retina, our results suggest that Hh...
factors, secreted from the retina itself, are involved in and necessary for the proper RPE cell differentiation. Finally, this analysis also allowed us to reveal some components of the Hh signalling as putative retinal stem cell markers.

**Hh genes are expressed in different cell types during retinogenesis**

We found that X-shh is expressed in newborn ganglion cells. This is consistent with previous data in mouse and zebrafish (Wallace and Raff, 1999; Stenkamp et al., 2000). We found that X-bhh and X-chh are expressed in the retina from stage 35 onwards, in the RPE but not in the neural retina. This expression seems to be maintained as detected by RT-PCR in the *Xenopus* adult retina (Takabatake et al., 1997). *Ihh* has also been detected, using RT-PCR, in the rat RPE (Levine et al., 1997). However, it has been reported that *Dhh* is expressed in the rat neural retina and not in the RPE, by RT-PCR (Levine et al., 1997). It seems therefore that the regulation in the retina of X-chh in *Xenopus* and of *Dhh* in mammals may have diverged during evolution.

**Complementary expression of Hh pathway genes in the central and the peripheral RPE**

Our data demonstrate a molecular difference between peripheral and central RPE cells in tadpole retina. Indeed, expression of X-bhh and X-chh is restricted to the central RPE cells, while X-Smo, Gli2 and Gli3 expression reveals a narrow peripheral annulus in the RPE. In addition, we found a zone between these two domains of expression that do not express any of these genes strongly. This therefore led us to propose a molecular subdivision of the RPE into three zones. From most peripheral to most central RPE, we found Hh downstream genes in the first zone, then these genes were inactivated, whereas Hh genes are not yet activated in zone 2. Next, Hh and Xotx5 genes were strongly activated in zone 3 (see Fig. 9A). This subdivision may not reflect distinct cell types as much as it does a gradient of differentiation from the periphery to the central RPE, similar to the gradient of differentiation that occurs in the CMZ (Perron et al., 1998). Antibodies against the different components of the pathway may be necessary to reveal such a gradient at a protein level. Previous data suggest that all RPE cells were not equivalent. Layer and Willbold have found that peripheral RPE behave differently from the central RPE in culture (Layer and Willbold, 1989). Indeed, it has been shown that retinal and pigmented cells have the ability to generate histotypic in vitro retina in culture. However, the sequence of layers is identical with that of in situ retina only if the pigmented cells are derived from the eye periphery (Layer and Willbold, 1989). In addition, our BrdU experiments suggests that pigmented cells in the peripheral epithelium are ‘younger’ than RPE cells in the central region as some peripheral RPE cells are still dividing while all central RPE cells are postmitotic in stage 42 embryos. This is consistent with the conventional idea of how the RPE grows. The central area differentiates earlier than the marginal zones (Stroeva and Mitashov, 1983). The proliferative state of the RPE has, however, led to debates (reviewed by Stroeva and Mitashov, 1983). In chick, it was assumed that mitotic activity had ceased completely in the RPE of a 4-day-old embryo, while others have found mitosis later in the embryonic RPE, but it was thought that by day 14, there were no dividing cells in the RPE. Recently, however, Fisher and Reh have re-examined the mitotic state of the retinal margin of hatched chicks (Fischer and Reh, 2000; Fischer and Reh, 2001). Surprisingly, they found a proliferative margin similar to the CMZ of amphibians, suggesting the presence of stem cells. In addition, consistent with our results, they found the presence of proliferative cells in the peripheral pigmented epithelium (Fischer and Reh, 2001). It is interesting in this context to note that retinal stem cells have been found in the pigmented ciliary body located in the margin of adult mouse retina. Although these cells do not have the capacity to regenerate in vivo, they can proliferate in vitro and differentiate into retinal-specific cell types, including rod photoreceptors, bipolar neurones and Müller glia (Tropepe et al., 2000; Ahmad et al., 2000). Therefore, the presence of retinal stem cells in the peripheral pigmented epithelium might be conserved in amphibians, chick and mammals. It is interesting to note that X-smo, Gli2 and Gli3 are strongly expressed only in retinal stem cells of the CMZ, (the most peripheral part of the CMZ) and in the peripheral pigmented epithelium. These genes are therefore the first markers of this retinal stem cell region. Indeed, genes that have been shown to be expressed in retinal stem cells so far in *Xenopus* CMZ are also still expressed in differentiating cells of the retina, such as *Pax6, Xoptx2* or *Rx1* (Perron et al., 1998; Zuber et al., 1999). However, genes expressed only in the CMZ, such as the bHLH gene *Xath5*, are not expressed in the most peripheral region

![](image.png)
containing stem cells (Kanekar et al., 1997; Perron et al., 1998). It would be interesting now to look at the expression of Smo or Gli genes in mammalian retina to see whether they also represent specific markers of pigmented ciliary margin retinal stem cells. It is now necessary to ask whether these peripheral pigmented epithelium cells in Xenopus retina do indeed self-renew and behave as retinal stem cells in vivo.

A role for X-shh in the proximodistal axis of the retina

At early stages of Xenopus eye formation, the proximal and ventral axes of the eye vesicle are somewhat confounded, and this is because the optic stalk, which is closer to the midline than the retina, is also continuous with the ventral retina in fate-map studies (Eagleson et al., 1995). Moreover, cells from the optic stalk migrate into the ventral retina during optic vesicle formation (Holt, 1980). Later, it is easier to differentiate between the proximodistal axis and the dorsoventral axis of the retina. The optic stalk, for example, expresses Pax2, which is a good marker for the proximodistal axis as it is expressed in the optic stalk but not the retina, while Vax2 is a good dorsoventral marker as it is expressed in the ventral but not the dorsal retina (Macdonald et al., 1995; Barbieri et al., 1999). As has previously been shown in zebrafish (Macdonald et al., 1995; Ekker et al., 1995b), our results suggest that X-shh, probably from the rostral midline, is involved in patterning the proximodistal axis of the optic vesicle (see Fig. 9B). Indeed, we observed an enlargement of the optic stalk at the expense of the neural retina when we overactivated the Hedgehog pathway and the opposite cyclops-like phenotype, with neural retinal cells in the optic stalk region, when we blocked the pathway using cyclopamine. Although the morphology of the retina is severely affected, retinal cells, including ganglion and photoreceptor cells, are born and the dorsoventral axis of the retina appears unaffected although we used only the one marker, Vax2. This is different from what has been seen in zebrafish (Neumuen and Nusslein-Volhard, 2000), where cyclopamine blocked neural retinogenesis. This might reflect a difference in the role of the Hh pathway between fish and amphibians. In agreement with our results, retinal ganglion cells develop normally in mice carrying a conditional mutation in the Shh locus (Wang et al., 2002). A role for the Hh pathway in the dorsoventral axis has also been proposed in chick (Zhang and Yang, 2001b). However, as we found that the dorsoventral border of the retina seems to be well established in cyclopamine-treated embryos, we conclude that the severe ventral phenotype is the result of only proximodistal axis defects. Interestingly, we found that embryos treated with cyclopamine only from the neurula stage (i.e. when the eye field separation has already occurred) still display proximodistal axis defects. This suggests a continuing role of Hh signalling in the proximodistal axis during the optic vesicle maturation.

A role for Hh genes in RPE cell differentiation

We have shown that blocking Hh signalling by cyclopamine, after the eyes have separated, does not lead to any obvious defect in neural retinal histogenesis. Rather, we found that cyclopamine induces severe RPE defects (see Fig. 9B). As we obtained RPE defects with a low dose of cyclopamine and with KAAD-cyclopamine, which has been shown to be less toxic but more potent, we could rule out a possible toxic effect. Similar to our results, when the Shh signal was perturbed only after the optic cup formation in chick embryos, using a blocking antibody, pigmentation was also affected, the ventral part being lost (Zhang and Yang, 2001b). We also found that the ventral part of the RPE is often more affected but we found that this was mainly due to a proximodistal axis defect. The RPE differentiation defect we have observed, however, occurs both in the dorsal and the ventral parts. One explanation for this difference may be that cyclopamine blocks all Hh signals while the anti-Shh antibody blocks only Shh signalling. Our expression data of Hh genes during normal retinogenesis is consistent with a role in RPE differentiation, X-shh being expressed in early RPE tissue, while X-bhh and X-chh are expressed later in RPE cells. As cyclopamine acts via Smoothened (Taipale et al., 2000; Chen et al., 2002), our data strongly suggest that the effect of Hh genes on RPE differentiation involves the Patched-Smoothened-Gli signalling cascade. Again, this is consistent with our finding that Ptc, Smo and Gli genes in Xenopus are indeed expressed in RPE precursors. Ptc1 is also expressed in RPE in chick optic vesicle (Zhang and Yang, 2001b). In tadpole embryos, Hh genes are expressed in differentiated RPE cells while cells that receive the signal are expressed in the young RPE cells including dividing cells of the peripheral pigmented epithelium. Moreover, our results suggest that Hh signal might instruct these cells to differentiate into mature RPE tissue. We could therefore make a parallel with what happens in Drosophila eye disc where differentiated photoreceptors behind the morphogenetic furrow express hh and instruct precursor cells to differentiate, allowing the progression of the furrow (reviewed by Burke and Basler, 1997).

Long-range effect of Hh signalling

In tadpole retina, we found that some cells, in between the domains of expression of X-bhh/X-chh and of Xsimo/Gli2/Gli3, do not seem to express any of these genes, thereby leaving a gap between cells secreting Hh and cells that potentially mediate the signal. This would be consistent with a long-range morphogen action that has been demonstrated in several other tissues (reviewed by Ingham and McMahon, 2001). In the vertebrate limb for example, Shh protein can spread for many cell diameters (Lewis et al., 2001). In tadpole embryos, we found a very low expression of Gli1, X-Ptc-1 and X-Ptc-2 in the RPE. As these three genes are transcriptionally regulated by the Hh pathway, this may reflect a weakly active pathway in tadpole embryos consistent with the low rate of de novo RPE cell production at this stage, compared with earlier development where we have detected a stronger expression of these three genes in the differentiating RPE. Altogether, these results suggest that Hh signalling, probably involving X-shh most at early stages and both X-bhh and X-chh at later stages, is required for generating RPE cells during retinogenesis in an ongoing process of central-to-peripheral axis formation in the growing eye.

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