Sonic hedgehog, secreted by amacrine cells, acts as a short-range signal to direct differentiation and lamination in the zebrafish retina

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Summary

Neurogenesis in the zebrafish retina occurs in several waves of differentiation. The first neurogenic wave generates ganglion cells and depends on *hedgehog* (*hh*) signaling activity. Using transgenic zebrafish embryos that express GFP under the control of the *sonic hedgehog* (*shh*) promoter, we imaged the differentiation wave in the retina and show that, in addition to the wave in the ganglion cell layer, *shh* expression also spreads in the inner nuclear layer. This second wave generates amacrine cells expressing *shh*, and although it overlaps temporally with the first wave, it does not depend on it, as it occurs in the absence of ganglion cells. We also show that differentiation of cell types found in the inner and outer nuclear layers, as well as lamination of the retina, depends on *shh*. By performing mosaic analysis, we demonstrate that Shh directs these events as a short-range signal within the neural retina.

Movies available online

Key words: Sonic hedgehog, Atonal homolog 5, Retina, Differentiation, Neurogenesis, Zebrafish

Introduction

During vertebrate eye development, seven major cell types are born in the retina and are arranged in a specific pattern in three layers. Owing to the accessibility of the developing eye to experimental observation and manipulation, the generation of this pattern has been the subject of numerous studies (reviewed by Livesey and Cepko, 2001). Recently, the zebrafish has gained popularity as a model for studying this process because of the availability of a number of experimental tools in this system (reviewed by Malicki, 2000; Neumann, 2001).

The Hedgehog (Hh) family of secreted signaling proteins participates in patterning the zebrafish retina. One of the earliest functions of Hh signaling is to induce optic stalk tissue at the expense of neural retina (Ekker et al., 1995; Macdonald et al., 1995; Perron et al., 2003). Slightly later, neurogenesis is initiated in the retina by a signal originating from the optic stalk (Masai et al., 2000). The first neurons to be born are retinal ganglion cells (RGCs), and these express *sonic hedgehog* (*shh*). From this nucleation point, a wave of RGC differentiation sweeps through the retina (Hu and Easter, 1999), and this wave requires *hh* signaling for its propagation (Neumann and Nuesslein-Volhard, 2000). This role of *hh* signaling in zebrafish retinal neurogenesis is reminiscent of the role of Hh in directing the retinal neurogenic wave in *Drosophila* (reviewed by Jarman, 2000; Kumar, 2001). A distinct wave of *shh* expression occurs in the retinal pigmented epithelium (RPE) and is associated with neurogenesis of photoreceptors in the outer nuclear layer (ONL) (Stenkamp et al., 2000; Stenkamp et al., 2002).

The bHLH transcription factor *atonal homolog 5* (*ath5*; *atoh7* – Zebrafish Information Network) is required for the generation of RGCs in the mouse and the zebrafish retina (Brown et al., 2001; Kay et al., 2001; Wang et al., 2001). In the zebrafish, *ath5* mutants lack RGCs completely, but have a thin ganglion cell layer (GCL) that is composed of 'misplaced' amacrine cells (Kay et al., 2001). *ath5* is expressed in a wave that precedes the wave of RGC differentiation (Masai et al., 2000), reminiscent of the way in which *Drosophila atonal* is expressed during eye development (Jarman et al., 1994; Dominguez, 1999).

Here, we show that *shh* expression spreads in an additional wave in the inner nuclear layer (INL), giving rise to a subpopulation of amacrine cells that express *shh*. By performing in vivo timelapse analysis, we show that this wave spreads almost simultaneously with the first wave in the GCL. However, the second wave is independent of the first wave, as it spreads normally in the absence of RGCs in *ath5* mutants. We also show that the differentiation of cell types found in the INL and ONL, as well as the laminar organization of the retina, depends on *shh* activity. By using mosaic analysis, we test which cells are the source of Shh in this context, and find that Shh directs these events as a short-range signal secreted by amacrine cells.

Materials and methods

Zebrafish mutant lines

The *shh* allele *syu*^{t4} has been described previously (Schauerte et al., 1998). The *ath5* allele *lak*^{th241} is described by Kay et al. (Kay et al., 2001).

Generation of *shh-GFP* transgenic line

Line *2.2shh:gfp:ABC#15* was produced by microinjection of the linear fragment of the construct *p2.2shh:gfp:ABC* (*Sal*I/*Kpn*I) containing the 2.2 kb upstream promoter (*Sal*I/*Xho*I fragment) from the *shh* locus (*pshh7*) (Chang et al., 1997), inserted upstream of *gfp* with a polyA signal followed by a *Not*I/*Kpn*I fragment of the *shh* locus (between +553 to +5631, AF124382) containing intron 1, exon 2 and intron 2. The promoter and intronic sequences contain all four enhancer regions required for activity in the floor plate and notochord (Müller et al., 1999). The integrated transgene is stably expressed in six subsequent generations, and is composed of head to tail concatemers of the transgene construct in an unknown number of copies (Y. Hadzhiev, unpublished).

Histochemical methods

In situ hybridization was performed on wholemounts, using *shh* as a probe (Krauss et al., 1993), a fluorescent signal was obtained by using Fast Red as a substrate for alkaline phosphatase (Roche), followed by confocal analysis. Antibody labeling was carried out on 12 μm thick cryosections and analyzed with a Leica confocal microscope. The following antibodies were used: rabbit anti-GFP (Torrey Pines Bioloabs, San Diego; 1:1000); mouse anti-Isl1 (Developmental Studies Hybridoma Bank; 1:50); mouse anti-Zn5 (University of Oregon; 1:500); mouse anti-Zpr1 (University of Oregon; 1:200); mouse anti-zpr3 (University of Oregon; 1:200); mouse anti-Glutamine Synthetase (BD Biosciences; 1:500); rabbit anti-cPKC βI (Santa Cruz Biotechnology; 1:200); and rabbit anti-GAD67 (Chemicon; 1:50). F-actin staining was carried out as described previously (Pujic and Malicki, 2001), using Alexa Fluor 568-conjugated phalloidin (1:40; Molecular Probes).

Transplantation

Transplantations were performed as described in Ho and Kane (Ho and Kane, 1990). Donor embryos transgenic for *shh-GFP* were injected at the one- to eight-cell stage with a 2.5% mixture of biotin- and rhodamine-conjugated dextrans (Molecular Probes) in a 1:9 ratio. Approximately 5-30 donor cells were transplanted into the animal pole of host embryos from a *syu* incross at late blastula stage. Host embryos were cryosectioned and analyzed by immunofluorescence, as described above. The biotin tracer was detected with Alexa Fluor 647-conjugated streptavidin (1:500; Molecular Probes) during the secondary antibody incubation.

Time-lapse analysis

Prior to imaging, *shh-GFP* transgenic embryos were manually dechorionated and transferred to embryo medium with 0.02% (w/v) tricaine. Embryos were then imbedded in a coverslip-bottomed petri dish (MatTek Corporation), in 0.5% low melting point agarose dissolved in embryo medium with 0.02% tricaine. After agarose polymerization, embryos were covered with embryo medium with 0.02% tricaine. Time-lapse imaging was performed on a LeicaNT confocal microscope using a HCPL APO 10×/0.040 IMM objective. Z series were obtained at 5 minute intervals with 8.8 μm steps over a total distance of 35.2 μm and then imported into ImageJ. A single z-plane was chosen and movies were compiled using the ImageJ software.

Results

Sonic hedgehog is expressed in a subset of RGCs and amacrine cells

To precisely define the cells which express *shh* in the zebrafish retina, we compared the retinal expression of *shh* RNA with a novel transgenic line expressing GFP under the control of the *shh* promoter (see Materials and methods). We find that both *shh* RNA and *shh-GFP* are expressed not only in the GCL, but also in the proximal part of the INL (Fig. 1A,D).

To further characterize the cells expressing *shh-GFP* in the retina, we compared the distribution of Zn5 antigen, which labels RGCs, with *shh-GFP* (Fig. 1B,C). The cells expressing *shh-GFP* in the INL display the typical morphology of amacrine cells (Fig. 1C). We also detect GFP in the inner plexiform layer (IPL), due to the presence of neurites from the *shh-GFP*-expressing amacrine cells (Fig. 1C).

We further compared *shh-GFP* expression with Isl1, a LIM/homeodomain transcription factor that is expressed in a range of neuronal cells, and has been detected in the GCL and in a subpopulation of amacrine cells in the rodent retina (Thor et al., 1991; Galli-Resta et al., 1997). We find that there are four types of RGCs detectable with this approach: those that express either *shh-GFP* or Isl1, those that express both, and those that express neither (Fig. 1G-I). We also find that the amacrine cells expressing *shh-GFP* in the INL are distinct from the amacrine cells expressing Isl1 (Fig. 1D-I).

These results indicate that *shh* is expressed in the GCL and in a subset of amacrine cells located in the proximal INL. This is in agreement with the observation that murine *Shh* is expressed both in the GCL and in the proximal INL (Jensen and Wallace, 1997). The amacrine cells expressing *shh* are distinct from the amacrine cells expressing Isl1.

In vivo imaging reveals that *shh* expression spreads in a wave in the INL that accompanies the RGC wave

In order to compare the spread of *shh* expression in the GCL and INL of the living retina, we imaged eyes of live transgenic *shh-GFP* embryos using confocal microscopy. *shh* expression is initiated at 28 hours post-fertilization (hpf) in the first RGC cells that differentiate in the ventronasal retina (Neumann and Nuesslein-Volhard, 2000). Although we detect *shh-GFP* protein with an anti-GFP antibody at 30 hpf (data not shown), GFP fluorescence is first detectable at 35 hpf (Fig. 2A, see movie at http://dev.biologists.org/supplemental/), due to the long folding time of GFP.

Following activation in the ventronasal patch, *shh-GFP* expression sweeps dorsally through the retina, reaching the dorsal margin by 46 hpf (Fig. 2F), 11 hours after the start of the wave. Interestingly, we observe the first amacrine cells expressing *shh-GFP* at 35 hpf, at the same time as when we detect the first RGCs expressing *shh-GFP* (Fig. 2A), and the spread of *shh-GFP* in the INL closely accompanies the RGC wave (Fig. 2A-F). Thus, the wave of *shh* expression starts in the INL well before it has reached completion in the GCL.

shh is expressed in a wave in amacrine cells in the absence of *ath5* activity

To test whether the *shh* wave in the INL depends on the *shh* wave in the GCL, we examined the spread of *shh* expression in the *lakritz* (*lak*) mutant, which disrupts the *ath5* gene and leads to the complete loss of RGCs (Kay et al., 2001). Although all *shh*-expressing RGCs are absent in *lak* mutants, we still detect *shh-GFP* expression in the inner retina of *lak* mutant embryos (Fig. 3A-D). These cells do not express the RGC marker Zn5 and are similar in morphology to the *shh-GFP*-expressing amacrine cells located in the wild-type INL, consistent with the observation that the formation of amacrine cells is not impaired by the loss of *ath5* (Kay et al., 2001). We observe the first *shh-GFP*-expressing amacrine cells in *lak* mutant embryos at 32 hpf, with an anti-GFP antibody (Fig.

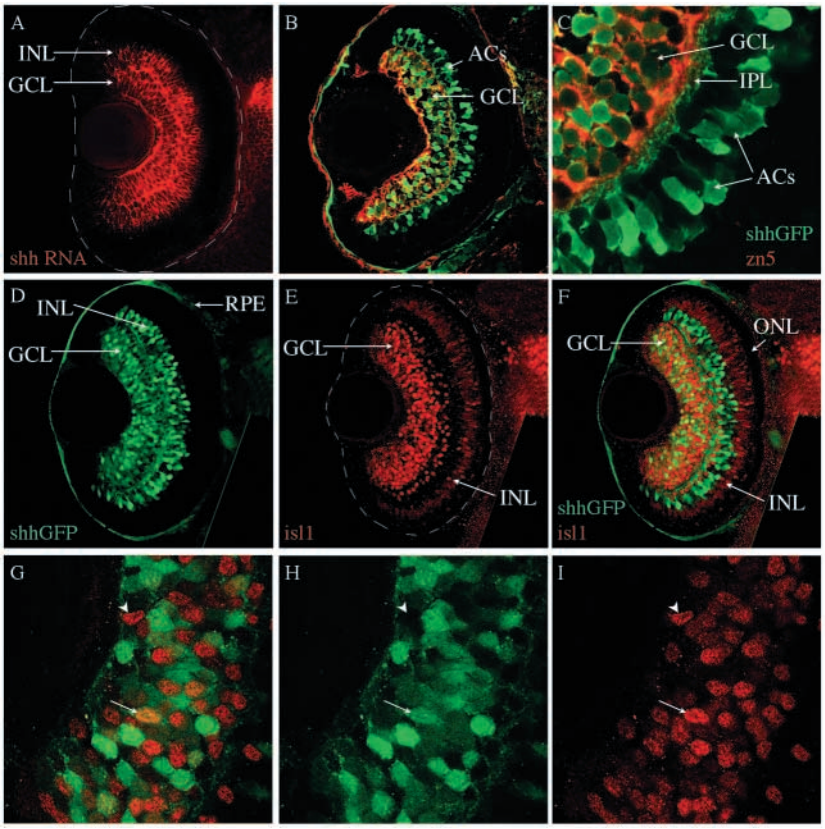


Fig. 1. Expression of *shh* in the zebrafish retina. All panels show confocal sections through the retina, with anterior to the top. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; ACs, amacrine cells. (A) Whole-mount detection of *shh* RNA in the retina at 46 hpf. Note expression in the GCL and proximal INL. (B) Expression of *shh-GFP* (green) and Zn5 (red). Note that *shh-GFP* and Zn5 are co-expressed in ganglion cells, and that *shh-GFP* is also expressed by a subset of amacrine cells. (C) High magnification of the retina shown in B. Note that GFP is also present in neurites that contribute to the inner plexiform layer (IPL). (D) Expression of the *shh-GFP* at 46 hpf (GFP single channel of the image shown in F). Note expression in the GCL, and proximal INL. (E) Isl1 single channel of the image shown in F. Note expression in the GCL and proximal INL, as well as in the distal INL. (F) Retina at 46 hpf, double labeled for Isl1 protein (red) and GFP (green) expressed by the *shh-GFP* line. (G) High magnification of the GCL double labeled for Isl1 (red) and *shh-GFP* (green) at 46 hpf. Note that some RGCs express only Isl1 (arrowhead), whereas some RGCs express both Isl1 and *shh-GFP* (arrow). (H) *shh-GFP* single channel of the image shown in G. (I) Isl1 single channel of the image shown in G.

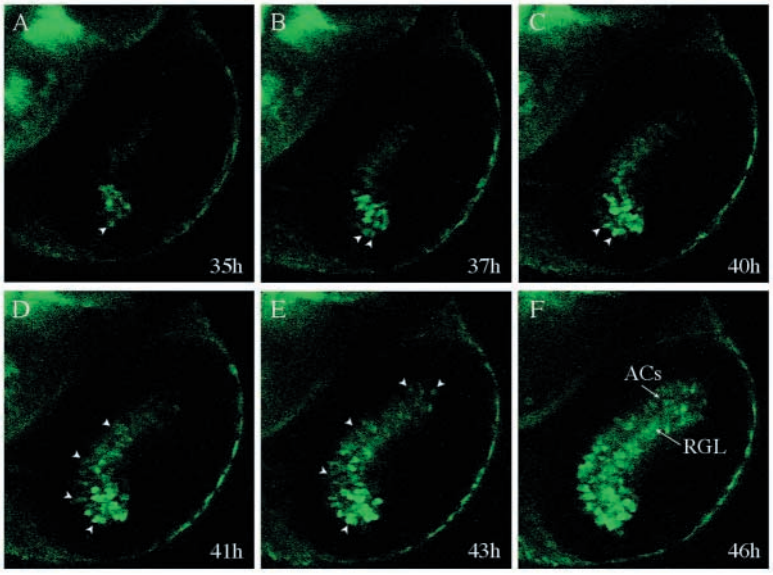


Fig. 2. In vivo time-lapse recording of *shh-GFP* expression in the retina. (A-F) Single frames taken from a film recording of *shh-GFP* expression in the retina, starting at 35 hpf and ending at 46 hpf. Note that the first amacrine cells expressing *shh-GFP* are already present at 35 hpf, and that the wave of *shh-GFP* expression in amacrine cells spreads rapidly following the wave in the GCL.

3C). At 32 hpf, we also detect a small number of *shh-GFP*-expressing cells in the wild-type retina that are not Zn5 positive (Fig. 3A, inset). These cells may be early-born amacrine cells that express *shh*, although it is also possible that they are a subset of RGCs that express *shh* earlier than Zn5. The *shh-GFP* wave sweeps through the *lak* retina by 48 hpf (data not shown), and *shh-GFP*-expressing amacrine cells are present both in the *lak* INL and the *lak* GCL (Fig. 3D), the latter corresponding to 'misplaced' amacrine cells that make up the *lak* GCL (Kay et al., 2001).

These results indicate that the wave of *shh* in the INL spreads independently of the RGC wave, and that *ath5* is not required for the spread of *shh* expression in amacrine cells. This wave of *shh* expression in amacrine cells has already started by 32 hpf, and fills the central retina by 48 hpf in *lak* mutant embryos, giving rise to amacrine cells in the INL and to misplaced amacrine cells in the *lak* GCL.

shh signaling is required for the differentiation of cell types found in the INL

As *shh* is expressed in the INL, does it also have a function in this layer of the retina? To address this question, we first examined cell types found in the INL in wild-type embryos and *shh* mutants.

In the rodent retina, Isl1 is expressed both in a subset of RGCs and in a subset of amacrine cells in the proximal INL (Thor et al., 1991; Galli-Resta et al., 1997). We find similar domains of Isl1 expression in the zebrafish retina (Fig. 1D, Fig. 4A). In addition, we also detect two distinct groups of Isl1-positive cells in the distal INL at 64 hpf that have not been described before, and that appear to be bipolar cells and

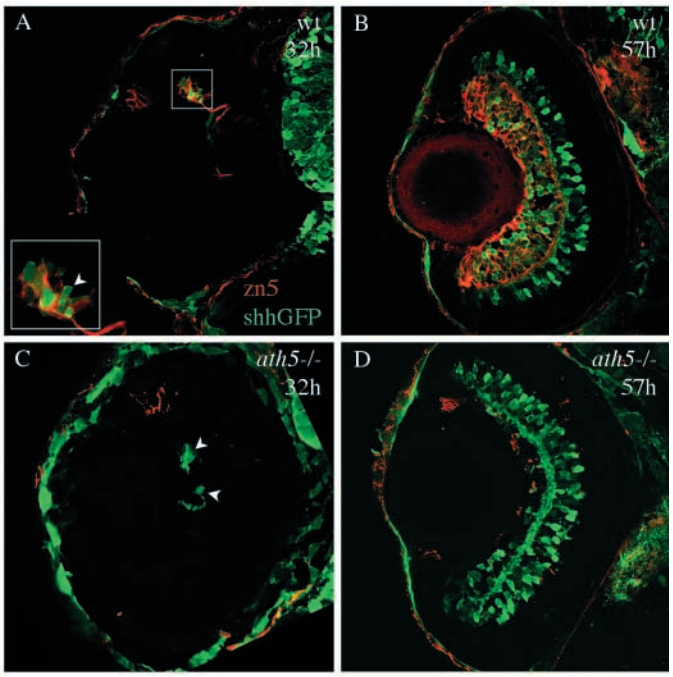


Fig. 3. A wave of *shh-GFP* expression spreads through the GCL and INL independently of *ath5* activity. All images are confocal sections of the retina, with anterior to the top. (A) Wild-type embryo double labeled for Zn5 protein (red) and *shh-GFP* expression (green) at 32 hpf. The expression of both markers has been initiated in the ventronasal sector. Note that some of the *shh-GFP*-expressing cells do not co-express Zn5, and are therefore amacrine cells (inset, arrowhead). (B) Wild-type embryo double labeled for Zn5 and *shh-GFP* at 57 hpf. Expression of both markers has spread through the retina. (C) *ath5* mutant embryo double labeled for Zn5 (red) and *shh-GFP* expression (green) at 32 hpf. Note the absence of Zn5-expressing cells, while a small number of *shh-GFP*-expressing cells are already present (arrowheads). (D) *ath5* mutant embryo double labeled for Zn5 and *shh-GFP* expression at 57 hpf. Note that *shh-GFP* expression has spread through both the GCL and the INL.

horizontal cells (Fig. 4A). In *shh* mutant embryos, we observe an overall reduction of Isl1-positive cells at 64 hpf, and most of these cells are found in the proximal retina (Fig. 4B). The number of Isl1-positive cells in the GCL of *shh* mutant embryos is greatly reduced compared with the wild-type GCL (Fig. 4A,B), consistent with the observation that the number of Zn5-positive RGCs is also strongly reduced in *shh* mutant embryos (Neumann and Nuesslein-Volhard, 2000). In addition, the other Isl1-expressing cell types are also strongly reduced or absent, and the laminar organization of Isl1-expressing cells is severely disrupted in *shh* mutants (Fig. 4B).

Mueller glia are a cell type whose cell bodies are found in the INL, and they express the marker glutamine synthetase (GS; Fig. 4C). We fail to detect GS immunoreactivity in *shh* mutant embryos at 72 hpf (Fig. 4D). At 96 hpf, we observe a very small number of GS-positive cells in *shh* mutants (data not shown), probably as a result of the activity of *tiggy winkle hedgehog* (*twhh*) (Neumann and Nuesslein-Volhard, 2000).

Bipolar cells are found in the distal INL, and we used protein kinase C (PKC) as a marker to detect the presence of

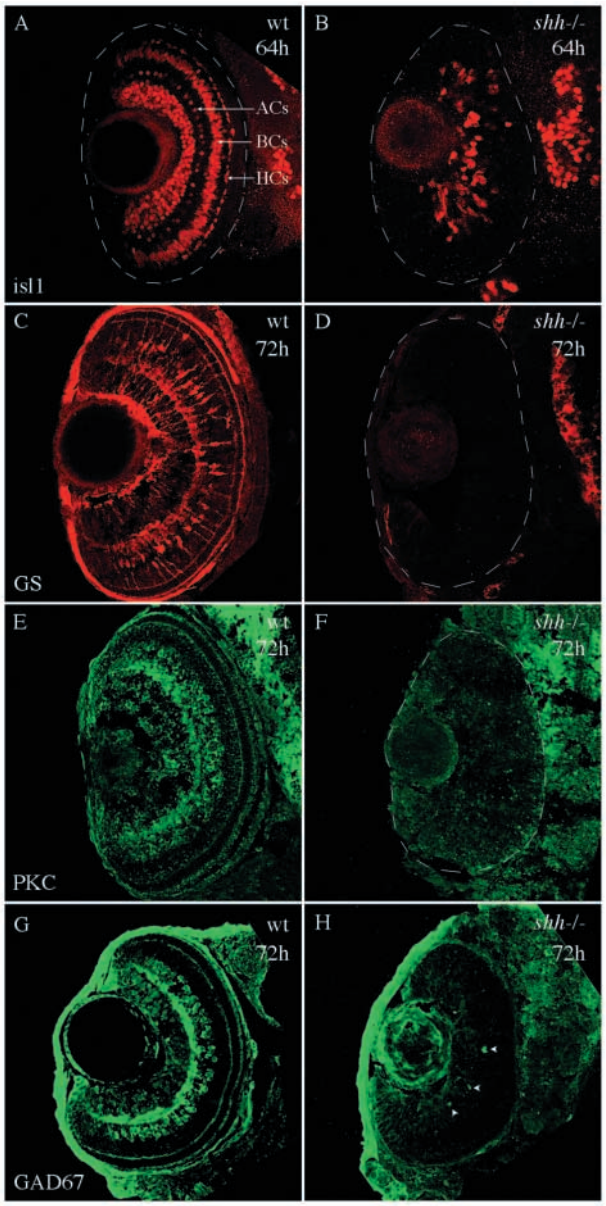


Fig. 4. The differentiation of cell types found in the INL depends on *shh* signaling. All images are confocal sections of the retina, with anterior to the top. (A) Isl1 protein (red) in a wild-type retina at 64 hpf. Note expression in the GCL, as well as in three discrete domains in the INL: amacrine cells (AC) in the proximal INL, as well as bipolar cells (BC) and horizontal cells (HC) in the distal INL. (B) Isl1 protein in a $shh^{-/-}$ retina at 64 hpf. Note the overall reduction of Isl1-expressing cells, and that most of them are located in the proximal retina. Note also that these cells are disorganized, and that there is no sign of an INL or laminar organization. (C) Glutamine synthetase protein (red) in a wild-type retina at 72 hpf. Note expression in Mueller glia, which span all the retinal layers. (D) Glutamine synthetase protein in a $shh^{-/-}$ retina at 72 hpf. Note the complete absence of glutamine synthetase staining from the retina. (E) PKC protein (green) in a wild-type retina at 72 hpf. Note prominent expression in bipolar cells in the INL. (F) PKC protein in a $shh^{-/-}$ retina at 72 hpf. Note the complete absence of PKC staining from the retina. (G) GAD67 protein (green) in a wild-type retina at 72 hpf. Note prominent expression in amacrine cells of the INL, and in the inner plexiform layer. (H) GAD67 protein in a $shh^{-/-}$ retina at 72 hpf. Note the severe reduction of amacrine cells (arrowheads) and absence of inner plexiform layer.

differentiated bipolar cells (Fig. 4E). We find no PKC-positive cells in the retina of *shh* mutant embryos at 72 hpf (Fig. 4F). This correlates well with the strong reduction of the Isl1-positive bipolar cells in *shh* mutant embryos (Fig. 4B).

Next we examined the expression of GAD67, which is a marker for differentiated amacrine cells (Fig. 4G). We detect only few GAD67-postive cells in the *shh* mutant retina, found in scattered locations, at 72 hpf (Fig. 4H).

Taken together, these results indicate that the differentiation of all cell types in the INL, including amacrine cells, bipolar cells, horizontal cells and Mueller glia, is dependent on *shh* activity.

shh is required for the generation of photoreceptors in the ONL, and for the formation of plexiform layers in the retina

It has previously been shown that the number of rod and cone photoreceptors is reduced in *shh* mutant embryos (Stenkamp et al., 2000; Stenkamp et al., 2002). As described, we see a dramatic reduction of Zpr1-expressing red/green double cones in *shh* mutant embryos at 72 hpf (Fig. 5C,D). Similarly, we also see a dramatic reduction of Zpr3-expressing rod photoreceptors at 72 hpf (Fig. 5E,F). These cells are present as a small cluster in a ventronasal patch, which is the region where photoreceptor differentiation commences in the zebrafish retina. The photoreceptor cells in *shh* mutants are sometimes restricted to their normal position in the ONL, but are also often found scattered in more proximal locations of the retina (data not shown). At 96 hpf, we detect a small increase in the number of photoreceptors in *shh* mutants (data not shown), which could be due to the activity of *twhh*.

We also wished to determine whether *shh* is required for the formation of the plexiform layers in the retina, and thus used phalloidin staining to reveal the laminar organization of the retina. Phalloidin stains F-actin, which is located predominantly on the inner surface of the plasma membrane and is present at elevated levels in regions of the retina with a high density of cell membranes, including the inner plexiform layer (IPL) and the outer plexiform layer (OPL; Fig. 5A). We do not detect any plexiform layers in *shh* mutant embryos at 50 hpf (Fig. 5B). We also examined the apical localization of mitotic nuclei in the retinal epithelium of *shh* mutants using an antibody recognizing γ-tubulin, but found no difference to wild-type embryos at 28 hpf (data not shown).

We observe a similar disruption of retinal differentiation and lamination when we treat embryos with the small molecule SANT-1 (data not shown), which has been shown to act as an antagonist of the Hh signal transduction pathway (Chen et al., 2002), further supporting the proposal that Hh signaling is crucial for these events.

These results indicate that *shh* activity is required for the differentiation of photoreceptors, as well as for the formation of plexiform layers in the retina.

shh acts as a short-range signal in the neural retina to direct differentiation and lamination

To determine which *shh*-expressing cells direct differentiation in the INL and the ONL, as well as the formation of plexiform layers, we transplanted wild-type cells into *shh* mutant embryos. We marked wild-type cells by injecting biotin-conjugated dextran into donor embryos; the donor embryos were simultaneously transgenic for *shh-GFP*, to permit the identification of *shh*-expressing cells in the wild-type clones.

Wild-type cells are able to restore the differentiation of Mueller Glia in the *shh* mutant retina (compare Fig. 6 with Fig. 4D). The rescued Mueller Glia express glutamine synthetase and show the elongated morphology of this cell type, spanning the whole retinal epithelium. We only observed rescue in *shh* mutant cells that were located in close vicinity to wild-type cells (Fig. 6B-D). Also, we only observed rescue in those regions of the retina in which wild-type cells expressing *shh-GFP* were found (Fig. 6A-D), indicating that *shh* functions as a short-range signal (n=16 eyes). Due to this observation, we also performed transplantations in which donor cells were

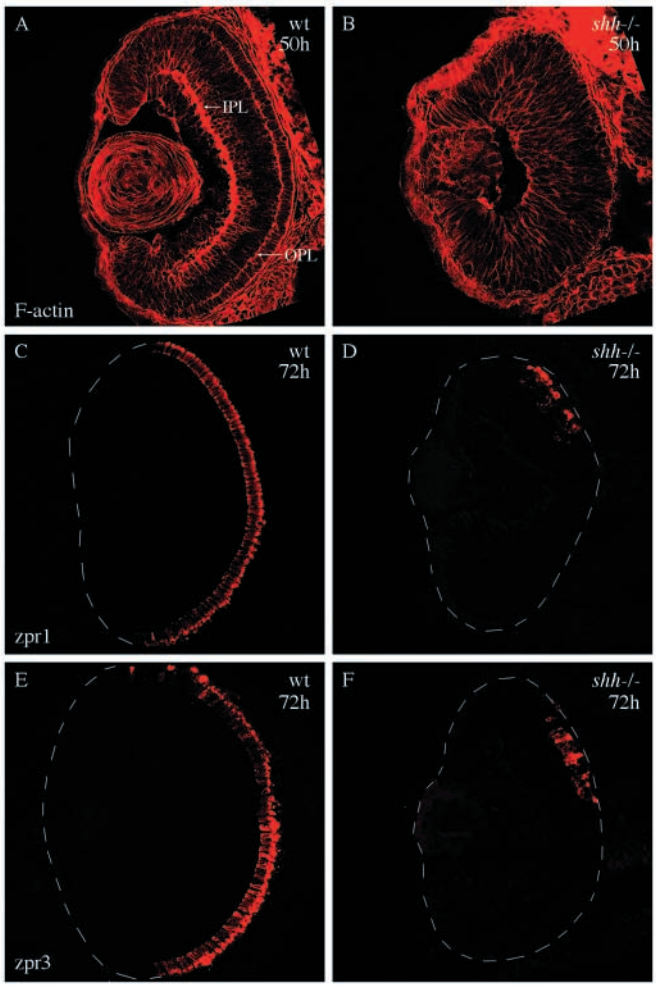


Fig. 5. The formation of plexiform layers and the differentiation of photoreceptors depends on *shh* signaling. All images are confocal sections of the retina, with anterior to the top. (A) F-actin staining in a wild-type retina at 50 hpf. Note the prominent staining of the inner and outer plexiform layers. (B) F-actin staining in a $shh^{-/-}$ retina at 50 hpf. Note the complete absence of plexiform layer formation. (C) Zpr1 protein in a wild-type retina at 72 hpf. Note the staining in cones of the ONL. (D) Zpr1 protein in a $shh^{-/-}$ retina at 72 hpf. Note the severely reduced number of cells expressing Zpr1, which are present in a ventronasal patch. (E) Zpr3 protein in a wild-type retina at 72 hpf. Note the staining in rods of the ONL. (F) Zpr3 protein in a $shh^{-/-}$ embryo at 72 hpf. Note the reduced cell number, as observed with Zpr1 (E).

transgenic for *shh-GFP*, but were not labeled with biotin-dextran. Wild-type cells were thus identified by virtue of their *shh* expression in the host retina. Again, we only observed rescue in the immediate vicinity of cells expressing *shh-GFP* in the neural retina (*n*=20 eyes) (Fig. 6E,F). In most cases, wild-type clones gave rise to both RGCs and amacrine cells expressing *shh*, but in a few cases, only amacrine cells expressing *shh-GFP* were present. We also observed rescue of Mueller Glia in those cases (Fig. 6D). We never observed rescue when *shh-GFP*-expressing cells were located in the pigmented retina (Fig. 6G).

Wild-type cells also rescue the differentiation of photoreceptors in the *shh* mutant retina, as assayed by the expression of Zpr1 (compare Fig. 7 with Fig. 5D) and by cellular morphology (Fig. 6B). Rescue was observed in mutant cells located close to wild-type cells, and in all cases, rescue of photoreceptors was only observed when *shh-GFP*-expressing cells were present in the neural retina (*n*=50 eyes) (Fig. 7A-E). However, no rescue was observed when *shh-GFP*-expressing cells were present only in the pigmented retina (Fig. 7F).

To determine the effect of *shh*-expressing wild-type cells on lamination in the *shh* mutant retina, we stained mosaic eyes with phalloidin. Rescue of the IPL was observed only in the immediate vicinity of *shh-GFP*-expressing cells in the neural retina (*n*=52 eyes) (compare Fig. 8A-F with Fig. 5B). If only a small number of transplanted wild-type cells expressed *shh-GFP*, we only observed rescue right next to these cells (Fig. 8G). Clones of wild-type cells failed to rescue lamination if they did not contain any *shh-GFP*-expressing cells (Fig. 8H). Likewise, lamination was not rescued if wild-type cells were located only in the pigmented retina (Fig. 8I,I′).

Taken together, these results indicate that wild-type *shh*-expressing cells located in the neural retina can rescue lamination and differentiation in the INL and ONL of *shh* mutants. This rescue only occurs close to the source of *shh* expression, indicating that Shh acts as a short-range signal in the neural retina to control differentiation.

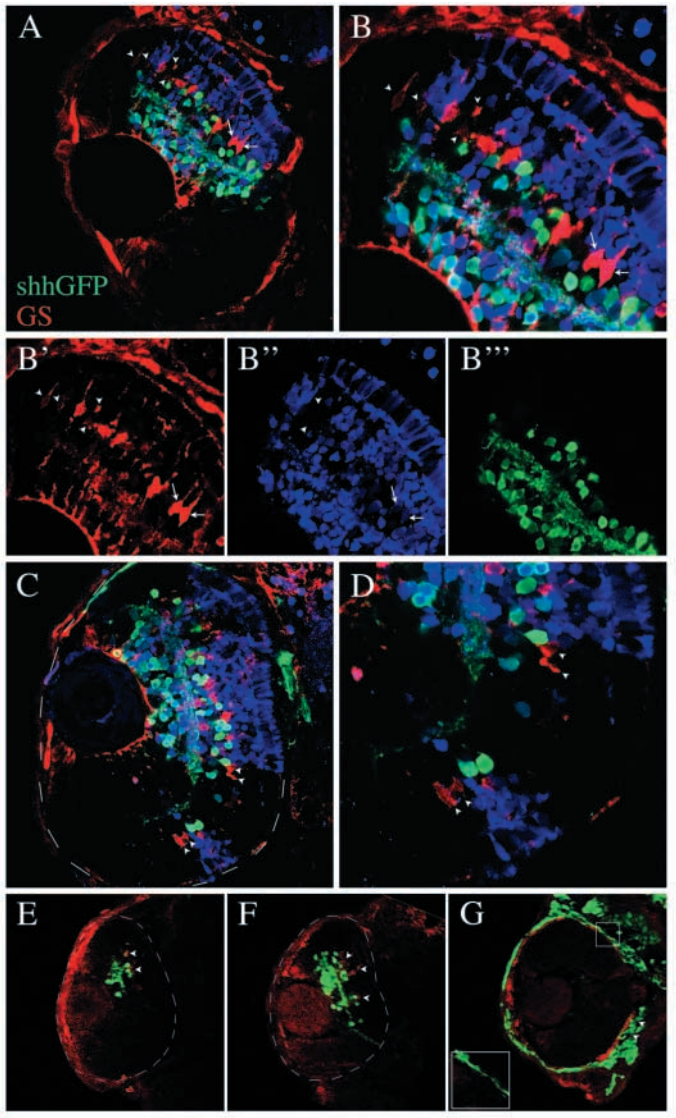


Fig. 6. Non-autonomous rescue of Mueller glia at 72 hpf by wild-type cells transplanted into *shh* mutant embryos. Wild-type cells are labeled with biotin (blue) and they also carry the *shh-GFP* transgene (green). Mueller glia are detected with an antibody recognizing Glutamine Synthetase (GS, red). (A) Mueller glia are rescued in the immediate vicinity of *shh*-expressing wild-type cells. The arrowheads point to *shh* mutant cells that express GS. Arrows indicate wild-type donor cells that express GS. (B) Higher magnification of the image shown in A. (B′-B‴) Single channels of the image shown in B. Arrowheads point to mutant cells that are rescued. (C) Another example showing non-autonomous rescue of mutant cells (arrowheads). Note only two *shh-GFP*-expressing wild-type cells are present in the INL in the lower portion of the retina, and that these cells rescue nearby mutant cells. (D) Higher magnification of the image shown in C. (E) An example of a small clone of wild-type cells expressing *shh-GFP* in the neural retina that leads to local rescue of Mueller glia (arrowheads). (F) A slightly larger clone of wild-type cells expressing *shh-GFP* that leads to local rescue of Mueller glia (arrowheads). (G) An example of failure to rescue by wild-type cells expressing *shh-GFP* present in the pigmented retina (RPE, arrowheads). (Inset) Enlarged view of framed area.

Discussion

The generation of neurons in the zebrafish retina occurs in several waves of differentiation that spread through the retinal epithelium. The first of these waves gives rise to RGCs, and both *ath5* and *shh* have been implicated in the regulation of this wave. Here, we have shown that *shh* is also expressed in a wave in the INL, giving rise to a subset of amacrine cells expressing *shh*, and that this wave is independent of RGCs and *ath5*. Furthermore, *shh* activity in the INL is required for the differentiation of amacrine cells, bipolar cells, Mueller glia and photoreceptors. Shh acts as a short-range signal in the neural retina to direct the differentiation of these cell types, as well as the formation of plexiform layers.

shh spreads in a wave in the INL following the GCL wave very rapidly

By performing in vivo timelapse recording on *shh-GFP* transgenic embryos, we were able to follow the spread of *shh* expression in the retina of living embryos. The most surprising result of this experiment was the observation that the first *shh*-expressing amacrine cells are already present one to two hours after the first *shh*-expressing RGCs can be detected, and that the wave of *shh* expression in the INL occurs almost

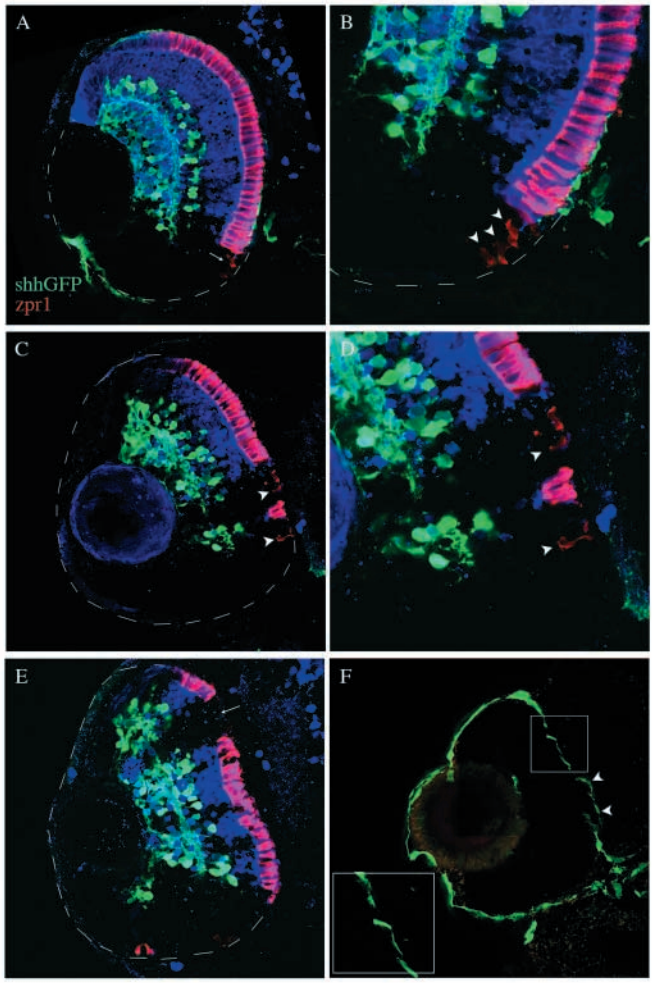


Fig. 7. Non-autonomous rescue of photoreceptors at 72 hpf by wild-type cells transplanted into *shh* mutant embryos. Wild-type cells are labeled with biotin (blue) and they also carry the *shh-GFP* transgene (green). Photoreceptors are detected with an antibody recognizing Zpr1 (red). (A) Photoreceptors are rescued when they are found in the region of the retina containing wild-type *shh*-expressing cells in the neural retina. The arrow indicates *shh* mutant cells that are rescued. (B) Higher magnification of the image shown in A. Arrowheads point to non-autonomously rescued cells. (C) Another example of the rescue of photoreceptors. (D) Higher magnification of the image shown in C. Arrowheads point to non-autonomously rescued cells. (E) A further example of rescue. Note that there is a gap in the rescued photoreceptors in exactly the same region as where there are no *shh*-expressing cells in the neural retina (arrow). (F) Example of failure to rescue photoreceptors when wild-type cells are located only in the RPE (arrowheads). (Inset) Enlarged view of framed area.

simultaneously with the wave of RGC differentiation. Previous studies based on BrdU labeling suggested that the first amacrine cells are born 10 hours after the first RGCs (Hu and Easter, 1999), and that the wave of INL differentiation is temporally distinct from the RGC wave. By contrast, our results indicate that the subset of amacrine cells that express *shh* are born very soon after the first RGCs, and that these cells spread in a wave that is temporally linked to the RGC wave. This conclusion is further supported by the observation that we observe the first *shh*-expressing amacrine cells in *ath5* mutants, which lack all RGCs, at 32 hpf, only a few hours after the RGC wave starts in wild-type embryos. Consistent with this, we also observe the first *shh-GFP*-expressing cells that are not Zn5 positive, and that are therefore not RGCs, at 32 hpf in the wild-type retina.

The wave of *shh* in the INL is independent of RGCs and independent of *ath5* activity

We observe the activation and spread of *shh* expression in amacrine cells in the absence of *ath5* activity, indicating that this wave of *shh* expression is independent of *ath5* and of RGCs. This is consistent with the finding that all cell types of the INL are formed in *ath5* mutant embryos (Kay et al., 2001). This wave of *shh* expression has already started in a ventronasal patch at 32 hpf, and spreads through the proximal region of the retina in the absence of *ath5* activity, giving rise to both the misplaced amacrine cells that are found in the GCL of *ath5* mutants, and the *shh*-expressing amacrine cells in the INL. These cells are likely to be the source of the Shh ligand that is required for the differentiation of INL cell types, as INL cell types differentiate normally in the absence of *ath5* activity, but not in the absence of *shh* signaling.

Control of cell differentiation in the INL by *shh* signaling

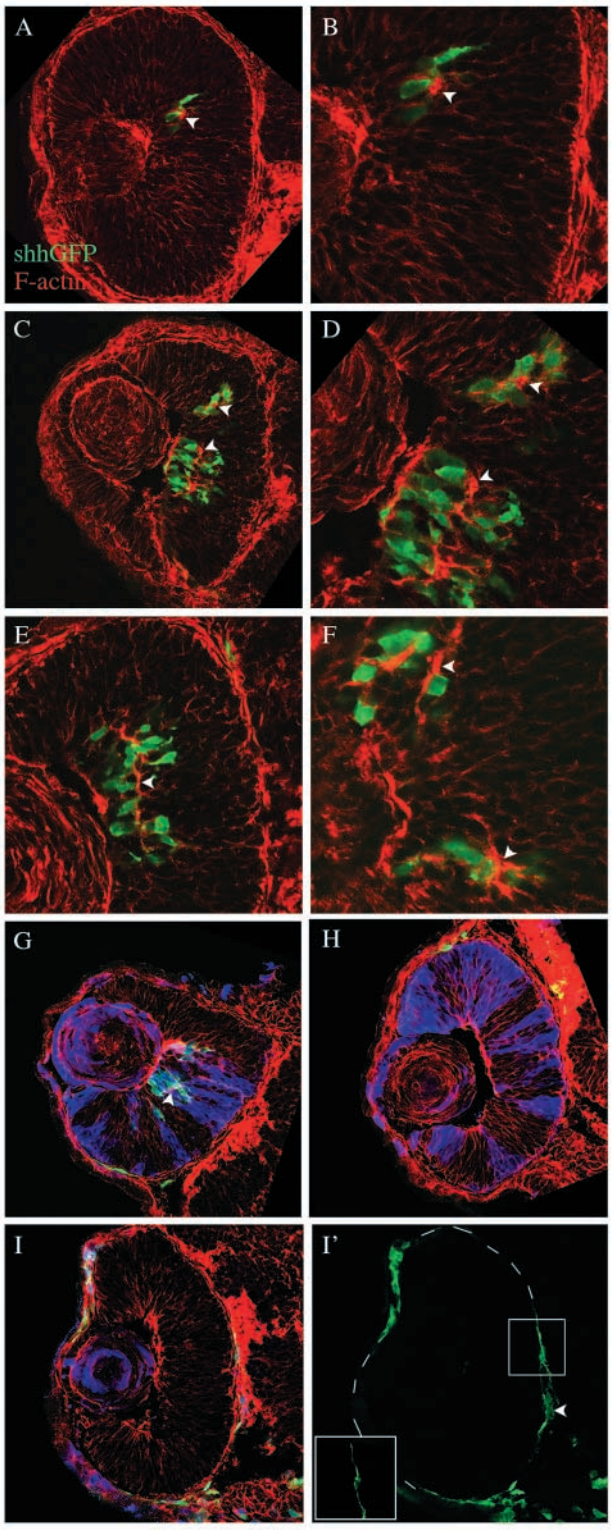
The work presented here indicates that *shh* signaling is required for the differentiation of all cell types found in the INL, including amacrine cells, bipolar cells, Mueller glia and horizontal cells. In fact, these cell types appear to be more sensitive to a reduction in *shh* signaling than RGCs are, as we have shown that the markers for differentiated bipolar cells (PKC) and Mueller glia (glutamine synthetase) are completely absent in *shh* mutant embryos, and a marker for differentiated amacrine cells (GAD67) is almost absent in *shh* mutant embryos. RGCs, however, are only partially depleted in *shh* mutant embryos and are completely lost only upon further reduction of Hh signaling with cyclopamine, probably due to the activity of *twhh*, which is also expressed in amacrine cells (Neumann and Nuesslein-Volhard, 2000) (and data not shown).

As *shh* is required for the differentiation of all major cell types in the retina, including glial cells, it does not appear to impart any information concerning which cell fate is adopted by the responding cells, and hence it appears to function simply as a differentiation promoting factor in the retina. We observe the loss of differentiated cells both in the central and peripheral regions of the retina in *shh* mutants (Fig. 4, and data not shown), indicating that Shh is required in both of these domains.

An important question in this context is how directly Shh signaling influences any of these cell types? An indirect mechanism of induction is suggested by the observation that several other signaling pathways have been implicated in controlling the differentiation of distinct cell types in the vertebrate retina. For example, activation of the Notch pathway has been shown to promote the differentiation of glial cells in the retina of frogs, rodents and zebrafish (Ohnuma et al., 1999; Furukawa et al., 2000; Scheer et al., 2001). This effect is cell-autonomous in the zebrafish, as even single cells in which the Notch pathway is activated assume a glial fate (Scheer et al., 2001). One possibility is thus that Shh signaling induces the

differentiation of Mueller glia by regulating the expression of Notch pathway ligands.

A further question that remains to be answered is at which stage Shh influences the transition from precursor cells to fully differentiated cells. Based on morphology, the cells remaining in *shh* mutants resemble precursor cells (data not shown), suggesting that they are blocked at a very early stage in differentiation. Unfortunately, we do not have molecular markers for intermediate stages of differentiation of the cells in the INL and ONL, and so cannot address this issue more directly.



Control of cell differentiation in the ONL and plexiform layer formation by *shh* signaling

Photoreceptor development in the zebrafish is severely impaired in the absence of *shh* activity (Stenkamp et al., 2000; Stenkamp et al., 2002) (this study). At 72 hpf, only a small, disorganized patch of cells expressing the photoreceptor markers Zpr1 and Zpr3 are found in a ventronasal patch in *shh* mutants, whereas the whole ONL is positive for these markers in wild-type embryos at the same stage. These findings complement those of Levine et al. (Levine et al., 1997) and Jensen and Wallace (Jensen and Wallace, 1997), who showed an increase in the expression of photoreceptor markers when retinal cultures of rat and mouse embryos were treated with Shh protein.

Our results also indicate that the plexiform layers fail to form in *shh* mutant embryos. The failure to form plexiform layers in *shh* mutant embryos is probably due to the almost complete absence of INL cell types in these embryos, as the axonal extensions of INL cells make important contributions to the plexiform layers.

Shh acts as a short-range signal in the neural retina to control retinal differentiation and lamination

To investigate which cells expressing *shh* are important for directing retinal differentiation, and to determine the range at which Shh controls differentiation and lamination, we performed mosaic experiments in which we transplanted wild-type cells into *shh* mutant embryos. We find that wild-type cells can rescue differentiation of Mueller glia and of photoreceptors in nearby mutant cells, but only when wild-type cells are located in the neural retina, and when they include *shh*-expressing cells in the GCL and/or INL. It has been suggested that *shh* expression in the RPE is responsible for directing photoreceptor differentiation (Stenkamp et al., 2000; Stenkamp et al., 2002), but our results do not support this proposal, because we observed rescue of photoreceptor development only when wild-type cells were located in the neural retina, but never when wild-type cells were found only in the RPE.

Fig. 8. Non-autonomous rescue of lamination at 50 hpf by wild-type cells transplanted into *shh* mutant embryos. Wild-type cells are unlabeled, but they carry the *shh-GFP* transgene (green). The inner plexiform layer is detected with phalloidin staining (red). (A) An example of a very small group of wild-type *shh*-expressing cells that lead to elevated levels of F-actin in adjacent cells (arrowhead) in the position where the IPL would normally form. (B) Higher magnification of the image in A. (C) Two groups of wild-type *shh*-expressing cells lead to local rescue of the IPL (arrowheads). (D) Higher magnification of the image in C. (E-G). Local rescue of the IPL by groups of wild-type cells expressing *shh*. In G, wild-type cells are labeled in blue, but rescue is only observed close to *shh-GFP*-expressing wild-type cells (arrowhead). (H) Large clones (blue) fail to rescue lamination if they do not contain *shh-GFP* expressing cells (green). (I) Wild-type cells expressing *shh-GFP* in the RPE fail to rescue lamination. (I′) The GFP single channel of the image shown in I, showing *shh-GFP*-expressing cells in the RPE (arrowhead, inset).

We have never observed a lateral spread of rescued cells more than a few cell diameters from cells expressing *shh*. Although this does not address the issue of whether the effect of Shh is direct (and it may well be indirect, caused by the activation of an intermediate signal or cell state), it indicates that this event is not propagated far from the source of Shh in the lateral plane of the retina. The further spread of rescued cells in the apical/basal plane of the retina may be either due to the fact that Shh, secreted by amacrine cells, affects precursor cells, which span the whole epithelium, or that the direct or indirect range of Shh is greater in this axis. In this context, it would be very useful to determine the range of Shh signaling in the retina. Unfortunately, because of the rapid spread of the differentiation wave, *patched*, which is a general target gene of Hh signaling, is only transiently expressed in the zebrafish retina, and as it is very difficult to obtain a robust signal by in situ, at the moment we cannot directly assay the range of Shh signaling in the apical/basal axis of the retina.

Most clones of transplanted wild-type cells form radial columns in the host retina, and thus include both RGCs and amacrine cells. However, in very rare cases, we observed the presence of wild-type *shh*-expressing amacrine cells only in the INL, and found that these cells are able to rescue nearby mutant cells. Taken together with the observation that the *ath5* mutant, which completely lacks RGCs, is able to generate all other cell types of the retina, this finding indicates that Shh secreted by amacrine cells is sufficient to direct differentiation in the zebrafish retina.

Formation of the IPL is rescued by wild-type cells expressing *shh* in the immediate vicinity, indicating that this effect of Shh on retinal lamination acts at very short range. The effect of Shh on plexiform layer formation is probably indirect, via its effect on cell differentiation. Restoration of lamination is thus likely to be due to the rescue of differentiated cell types in the INL and GCL that contribute to the IPL with their neurites. In this context, it is interesting to note that Shh has been shown to promote laminar organization in the murine retina by signaling to Mueller glia (Wang et al., 2002).

These results indicate that Sonic hedgehog plays a crucial role in the control of differentiation in the zebrafish retina. It will be interesting to determine the interplay between Sonic hedgehog signaling and other signals in orchestrating the birth and assembly of neurons and glia in the retina.

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