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Regulation of retinal ganglion cell production by Sonic hedgehog

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SUMMARY

Previous work has shown that production of retinal ganglion cells is in part regulated by inhibitory factors secreted by ganglion cell themselves; however, the identities of these molecules are not known. Recent studies have demonstrated that the signaling molecule Sonic hedgehog (Shh) secreted by differentiated retinal ganglion cells is required to promote the progression of ganglion cell differentiation wave front and to induce its own expression. We present evidence that Shh signals play a role to negatively regulate ganglion cell genesis behind the differentiation wave front. Higher levels of Shh expression are detected behind the wave front as ganglion cells accumulate, while the Patched 1 receptor of Shh is expressed in adjacent retinal progenitor cells. Retroviral-mediated overexpression of Shh results in reduced ganglion cell proportions in vivo and in vitro. Conversely, inhibiting endogenous Shh activity by anti-Shh antibodies leads to an increased production of ganglion cells. Shh signals modulate ganglion cell production within the normal period of ganglion cell genesis in vitro without significantly affecting cell proliferation or cell death. Moreover, Shh signaling affects progenitor cell specification towards the ganglion cell fate during or soon after their last mitotic cycle. Thus, Shh derived from differentiated ganglion cells serves as a negative regulator behind the differentiation wave front to control ganglion cell genesis from the competent progenitor pool. Based on these results and other recent findings, we propose that Shh signals secreted by early-differentiated retinal neurons play dual roles at distinct concentration thresholds to orchestrate the progression of retinal neurogenic wave and the emergence of new neurons.

Key words: Sonic hedgehog, Chick, Retina, Ganglion cells, Differentiation

INTRODUCTION

During vertebrate embryogenesis, the neural retina is derived from the inner layer of the optic cup, which originates from the anterior neural tube. The retina primordium contains proliferating progenitor cells that give rise to an intricate mature neural network consisting of seven neuronal and glial cell types (Dowling, 1987). The production of different retinal cell types follows a temporal sequence conserved among vertebrate species (reviewed by Altshuler et al., 1991). As elsewhere in the developing central nervous system (Edlund and Jessell, 1999; Lillien, 1998), control of retinal differentiation appears to involve the interplay of cell-extrinsic and cell-intrinsic factors. Cell lineage studies have demonstrated that vertebrate retinal progenitor cells are multipotent, i.e. capable of producing distinct progeny cells, suggesting the involvement of environmental influences in cell fate specification (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Turner et al., 1990; Fekete et al., 1994). Consistent with this notion, a variety of diffusible factors that influence retinal neurogenesis have been identified (Hicks and Courtois, 1992; Altshuler et al., 1993; Kelley et al., 1994; Lillien, 1995; Fuhrmann et al., 1995; Ezzeddine et al., 1997; McFarlane et al, 1998; Yourey, 2000). Cell culture studies have further revealed that retinal progenitor cells exhibit altered developmental potentials at different stages of neurogenesis, reflecting the progression of progenitor intrinsic properties (Watanabe and Raff, 1990; Watanabe and Raff, 1992; Alexiades and Cepko, 1997; Morrow et al, 1998; Belliveau and Cepko, 1999). Accumulating evidence also indicates that nuclear transcription factors play important roles for the competence as well as determination of retinal cell fates (Furakawa et al., 1997; Chen et al., 1997; Yan and Wang, 1998; Morrow et al., 1999; Kanekar et al., 1997; Perron et al., 1999; Liu et al., 2000). Thus, cell type specification and differentiation in the vertebrate retina is regulated by both cell-extrinsic cues present in the changing retinal environment and a repertoire of cell-intrinsic factors expressed by retinal progenitors (reviewed by Cepko et al., 1996; Harris, 1997; Reh and Levine, 1998; Cepko, 1999).

Among the cell-extrinsic factors, *Drosophila* Hedgehog (Hh) and its vertebrate homolog Sonic Hedgehog (Shh), emerge as crucial signaling molecules that regulate the development of the *Drosophila* compound eye and the vertebrate eye, respectively, despite morphological differences between the invertebrate and vertebrate visual systems. Active forms of the Hh family of proteins (Hh-N) mediate their signaling activities through a heteromeric receptor complex,

which includes the transmembrane Smoothened protein and the receptor Patched 1 (Ptc1) (reviewed by Hammerschmidt et al., 1997; Goodrich and Scott, 1998; McMahon, 2000). During mammalian eye primordium formation, *Shh* mutations cause severe cyclopia in mice and humans (Chiang et al., 1996; Belloni et al., 1996; Roessler et al., 1996; Ming et al., 1998), indicating a role for Shh signals in establishing the bilateral eye fields. Experimental manipulation of Shh signal levels in zebrafish, mouse, frog and chick have further demonstrated that Shh signals emanating from ventral midline tissues coordinate with other factors to determine the dorsoventral patterns of the retina and to influence compartmentalization of the optic cup (Macdonald et al., 1995; Ekker et al., 1995; Schulte et al., 1999; Hallonet et al., 1999; Koshiba-Takeuchi et al., 2000; Zhang and Yang, 2001). During retinal neurogenesis, exogenous Shh-N protein promotes rodent retinal progenitor cell proliferation, as well as differentiation of late arising cell types including photoreceptors in vitro (Jensen and Wallace, 1997; Levine et al., 1997). Reduction of zebrafish *shh* and *tiggywinkle hedgehog* (*twhh*) expression similarly results in the retardation of photoreceptor differentiation (Stenkamp et al., 2000). In addition, Shh produced by retinal ganglion cell axons stimulates astrocyte proliferation in the rat optic nerve (Wallace and Raff, 1999).

The secreted Hh protein plays fundamental roles in *Drosophila* compound eye development. At the onset of neurogenesis, Hh secreted from the posterior margin of the eye imaginal disc is required for the initiation of neuronal differentiation (Dominguez and Hafen, 1997; Pignoni and Zipursky, 1997), which proceeds in a posterior-to-anterior direction in the wake of the morphogenetic furrow (MF) that sweeps across the disc epithelium (Tomlinson and Ready, 1987; Wolff and Ready, 1993). Subsequently, Hh signals secreted from differentiated photoreceptor cells drives progression of the MF by recruiting additional cells anterior to the MF to enter a competent state for neurogenesis, and eventually to express Hh as new-born photoreceptor cells (Heberlein and Moses, 1995; Treisman and Heberlein, 1998; Greenwood and Struhl, 1999). In addition, Hh produced by the differentiated R8 photoreceptors controls ommatidial assembly through regulation of the proneural gene *atonal*, a bHLH transcription factor and a determinant of R8 cells (Jarman et al., 1994, White and Jarman, 2000). Genetic manipulation of Hh signaling has demonstrated that low levels of Hh signal occurring at a distance anterior to Hh producing cells act to induce the expression of *atonal*; while higher levels of Hh signal found in the vicinity of the newly differentiated ommatidial units suppress *atonal* expression between nascent proneural clusters, and thus critically control the position and number of future R8 cells (Dominguez, 1999).

Increasing evidence suggests that development of vertebrate retinal ganglion cells (RGC) resembles the development of the Drosophila R8 photoreceptor cells. Like the R8 cells, which serve as the founding cell of each ommatidium, RGCs are the first neurons to differentiate within the vertebrate retinal neural epithelium (Young, 1985; Spence and Robson, 1989; Altshuler et al., 1991; Prada et al., 1992; Snow and Robson, 1994). RGCs begin to differentiate at the ventricular surface of the retinal epithelium immediately after their terminal mitotic division (Waid and McLoon, 1995), and their cell bodies eventually occupy the inner layer of the retina with their axons extending through the optic nerve towards the brain. The differentiation of RGCs in the vertebrate retina initiates at the junction of the optic cup and the optic stalk, and spreads as a wave front towards the peripheral retina (Hu and Easter, 1999; McCabe et al., 1999; Masai et al., 2000). Although no cell cycle synchronization of progenitor cells ahead of the RGC wave front similar to the MF has been found, RGCs emerge at the front of the neurogenic wave in a non-random patterned array (McCabe et al., 1999). In addition, vertebrate homologs of the proneural bHLH transcription factor atonal are expressed in retinal progenitors and later in differentiating RGCs (Jasoni et al., 1994; Kanekar et al., 1997; Brown et al., 1998).

The molecular mechanisms that control the initiation of RGC differentiation and propel the RGC wave progression in vertebrate retina have begun to be elucidated. Zebrafish mutants of the Nodal signaling pathway, which lack axial mesoderm and consequently the optic stalk cells, fail to initiate expression of the vertebrate *atonal* homolog *ath5* in the neural retina (Masai et al., 2000), suggesting involvement of midline-derived signals in the initiation of RGC differentiation. Consistent with previous findings that FGF promotes the retinal neurogenic pathway (Guillemot and Cepko, 1992; McFarlane et al., 1998), blocking of FGF receptor activation in chick interferes with the movement of the RGC differentiation front (McCabe et al., 1999). Recently, *Shh* and *twhh* have been shown to be expressed in differentiated zebrafish RGCs (Neumann and Nuesslein-Volhard, 2000), as previously reported in the mouse retina (Jensen and Wallace, 1997). Moreover, Shh is necessary and sufficient to induce its own expression in new-born RGCs; *shh* mutations, as well as blocking Hedgehog signaling, retard the spread of the zebrafish RGC wave front (Neumann and Nuesslein-Volhard, 2000). These findings demonstrate a striking mechanistic conservation between the developing vertebrate and *Drosophila* eyes.

We address one central issue of retinal neurogenesis: the control of cell numbers of a given cell type. Current evidence suggests that specification of the RGC fate from the undifferentiated retinal neural epithelium involves mechanisms mediated by cell-cell contact and by secreted molecules. The transmembrane receptor Notch and its cell-surface ligand Delta are involved in cell fate specification of proliferating progenitors (Dorsky et al., 1995; Dorsky et al., 1997; Austin et al., 1995; Henrique et al., 1997; Ahmad et al., 1997; Bao and Cepko, 1997). An increased number of ganglion cells are produced either when early retinal progenitor cells are relieved of cell-cell contact or when Notch-mediated signals are reduced. Conversely, constitutive activation of the Notch receptor results in decreased ganglion cell production. Retinal culture studies indicate that retinas at later stages of neurogenesis contain secreted factors that inhibit retinal progenitor cells to differentiate into ganglion cells, and that these inhibitory activities are predominantly produced by ganglion cells themselves (Waid and McLoon, 1998). To date, molecules that mediate the negative feedback control on RGC production have not been identified. In this study, we have tested the hypothesis that Shh molecules produced by differentiated RGCs in the retina play a role in negatively regulating RGC production. We provide evidence that behind the RGC differentiation front, elevated levels of Shh result in a decrease in ganglion cell production, whereas reduced levels

of Shh lead to an increase of ganglion cells. We further demonstrate that Shh signals influence the progenitor-to-ganglion cell fate specification either during or soon after the last mitotic cell division. These findings, together with the recent demonstration on the role of Shh in controlling RGC wave progression (Neumann and Nuesslein-Volhard, 2000), lead us to propose that Shh signals secreted by differentiated ganglion cells play dual roles at the initial stages of vertebrate retinal neurogenesis in a similar fashion to those found for Hh during *Drosophila* compound eye development (Dominguez, 1999).

MATERIALS AND METHODS

Chick embryos

White Leghorn chicken eggs were purchased from Spafas and incubated at 38°C in a rotating humidified incubator. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Retroviral stocks and injections

The replication competent avian retrovirus RCAS(A).Shh was originally constructed and characterized by Riddle et al. (Riddle et al., 1993). The parental RCAS(A) virus (Hughes et al., 1987) was used as the control virus. Viral stocks with 1-2×10^8 cfu/ml titers were prepared by either transfecting chick embryonic fibroblast cells (CEFs) with viral DNA constructs, or infecting CEFs with viral stocks. Culture media were collected and concentrated by centrifugation as previously described (Morgan and Fekete, 1996).

Concentrated viral stocks were mixed with 1/10 volume of 0.25% Fast Green dye (Morgan and Fekete, 1996) immediately before injection. For stage 10 infections, the viral inoculum was injected into the neural tube at the junction of the forebrain and midbrain, as well as directly into the optic vesicles, until the primary optic vesicles were filled (0.2 to 0.4 μl). For stage 17-18 infections, the viral inoculum was delivered into the subretinal space between the retina and the pigmented epithelium layers of the right optic cup of embryos. Eggs were sealed with tape and further incubated in a stationary position at 38°C for designated periods before embryos were harvested.

Injection of hybridoma cells

Hybridoma cells producing anti-Shh IgG antibodies (5E1, Ericson et al., 1996) were obtained from the Developmental Studies Hybridoma Bank (DSHB, University of Iowa, Iowa City, IA) and grown in IMDM (Iscoves's Modified Dubecco's Medium, Gibco BRL) supplemented with 20% fetal calf serum and 2 mM glutamine. The control hybridoma cell line producing anti-viral GAG protein IgG antibodies (3C2, Stoker and Bissell, 1987) was cultured in DMEM supplemented with 10% fetal calf serum. For intravitreal eye injections, hybridoma cells were harvested by low speed centrifugation, followed by two washes in MEMAM (Minimum Essential Medium Alpha Modification, JRH) with 10 mM Hepes pH 7.0, and resuspended at 2×10^5 cells/μl in DMEM with 10 mM Hepes. Cells were mixed with 1/10 volume of 0.25% Fast Green dye immediately before injection. Approximately 0.1-0.2 μl of cells was injected into the vitreal space of the right eye of stage 17-18 embryos using pulled glass pipettes.

In situ hybridization

In situ hybridization was performed using 14 μm cryosections. Digoxigenin-labeled RNA probes were generated according to the manufacturer's instruction (Boehringer Mannheim). Chicken Shh (Riddle et al., 1993) and Ptc1 (Marigo et al., 1996) cDNAs were kindly provided by Dr Cliff Tabin and colleagues (Harvard Medical School). In situ hybridization procedures were as previously described (Yang and Cepko, 1996), except the hybridization for Shh was performed at 65°C overnight, followed by two 30 minute washes at 65°C in 50% formamide, 1×SSC and 0.1% Tween 20. Sections were then incubated with blocking solution and processed for anti-digoxigenin antibody incubation. For each probe, a minimum of three control or treated embryos were sectioned and analyzed.

Explant cultures

Two types of tissue explants were deployed. First, for most retinal explant culture experiments, stage 23 to stage 25 chick eyes were dissected such that the peripheral 25% of the eye (containing the lens and the ciliary margin) was discarded and only the center 75% of the retina without the pigmented epithelium was used. Second, for quantification of total cell number, stage 24 whole retinas including central and peripheral portions were used. Retinal explants were transferred on top of polycarbonate filter discs (Costar; Ezzeddine et al., 1997) and incubated at 37°C in a 5% CO_2 incubator for designated time periods floating on medium containing 42.8% DMEM, 50% F12, 1% fetal calf serum, 0.2% chick serum, 10 mM Hepes pH 7.0 and penicillin/streptomycin.

For antibody blocking experiments, antibodies were added to the culture medium at the beginning of the culture. The final concentrations for the anti-Shh-N antibody 5E1 (IgG, obtained from DSHB; Ericson et al., 1996) and the control anti-viral antibody 3C2 (IgG, obtained from DSHB; Stoker and Bissell, 1987) were 20 μg/ml. For viral infection experiments, 2 μl of viral stocks at 2×10^8 cfu/ml was diluted to 50 μl first and then added to retinal explants atop the filter. After a 36 hour culture period, the infection rates for the Shh virus and the RCAS virus were 60-80% and 30-40%, respectively, as assayed by immunostaining for the viral GAG protein using the monoclonal antibody 3C2 or the polyclonal antibody p27 (SPAFAS).

For in vitro BrdU labeling of explants, BrdU was added directly to culture media to a final concentration of 20 μM for designated time periods. For pulse-labeling experiments (shown in Fig. 8), stage 23 explants were treated with antibodies or viruses at the beginning of the culture period for 40 hours, then BrdU was added to the medium at 20 μM for 2.5 hours. Explants were then washed extensively and transferred to fresh filters and media for an additional 6 or 12 hours before dissociation and immunostaining.

Antibody sources

The anti-Islet 1 (4D5, Yamada et al., 1993), anti-Shh (5E1, Ericson et al., 1996) and anti-viral GAG protein (3C2, Stoker and Bissell, 1987) monoclonal antibodies were obtained from the DSHB. The polyclonal antibodies p27 against the viral GAG protein were obtained from SPAFAS. The monoclonal antibody recognizing the 68 kDa neurofilament (NF68) was purchased from Sigma, whereas polyclonal antibodies against the 145 kDa neurofilament (NF145) were obtained from Chemicon. The anti-β-tubulin polyclonal antibodies, which recognizes the same class III β-tubulin as the monoclonal antibody TUJ1, were purchased from Covance. The anti-BrdU antibody containing nuclease was obtained from Amersham. The anti-phosphohistone H3 antibody (PH3) was purchased from Upstate Biotechnology.

Immunocytochemistry and quantification

Staining of tissue sections with all antibodies was performed using 14 μM thickness cryosections of tissues fixed with 4% paraformaldehyde. Sections were incubated with primary antibodies and visualized using either biotinylated secondary antibodies and the Vectastain ABC Elite kit (Vector Laboratories), or fluorescent-labeled secondary antibodies. Horseradish peroxidase staining using 3′,3′-diaminobenzidine (DAB) as chromogen was visualized using Nomarski microscopy, whereas fluorescent signals were imaged by conventional fluorescent microscopy. For section immunostaining experiments, a minimal of three control eyes and three Shh virus- or 5E1 antibody-treated eyes of a given stage were analyzed.

Staining of dissociated retinal cells was performed as described by Altshuler and Cepko (Altshuler and Cepko, 1992). Briefly, retinas or retinal explants were incubated with trypsin followed by trituration in the presence of DNase to achieve single-cell suspensions, and then cells were plated on 10 μg/ml poly-D-lysine coated multi-well glass slides (Cel-Line Associates) at a density of 75,000 cells per 7 mm diameter well. Cells were further incubated at 37°C for one hour in retinal explant culture medium (see Explant cultures section) to allow attachment to the glass slides, followed by fixation and immunostaining. Bound primary antibodies were detected either by Texas Red-conjugated (Jackson ImmunoResearch Laboratories) or Alexa 488-conjugated (Molecular Probes) secondary antibodies with co-staining of cell nuclei by DAPI.

For in vivo labeling with BrdU, eggs were windowed and 100 μl of BrdU at 1 mg/ml were dripped on top of each embryo. The embryos were then returned to incubation for an additional 6 hours. Retinas were then either dissociated followed by staining as monolayer cells as described above for quantification, or fixed and embedded in paraffin. Sections were stained for BrdU incorporation as previously described (Beleycky-Adams et al., 1996).

To quantify total cell numbers per retina, retinas were individually dissociated as described above into single-cell suspensions. After dilutions, the numbers of cells in 0.1 μl were counted using a cytometer, and total number of cells per retina was calculated. Total numbers of BrdU-labeled cells were calculated based on percentages of marker-positive cells and total cell numbers determined as above. In general, percentages of marker-positive cells among total cells were determined by calculating the ratio of the fluorescent-labeled cells and the total DAPI-stained nuclei in a given dissociated cell sample. Double marker-positive cells were scored similarly. Student's *t*-test was used for statistical analyses; $P<0.05$ was considered statistically significant.

TUNEL assays

Cryosections (14 μm) were used in TUNEL assays (Gavrieli et al., 1992) with fluorescein-conjugated nucleotides (Boehringer Mannheim), according to the manufacturer's instructions. Apoptotic signals and cell nuclei stained by DAPI were visualized using epifluorescent microscopy.

RESULTS

Expression of Shh and its receptor Ptc1 in the early chick retinal neuroepithelium

In chick, rudimentary optic vesicles begin to form during neurulation (stage 8.5; 7 somites) and morphogenesis of the double-layered optic cup is complete by stage 17 (29-32 somites; Hamburger and Hamilton, 1951). However, the retina, which occupies the inner layer of the optic cup, does not begin differentiating until stage 20 (Spence and Robson, 1989; Snow and Robson, 1994; Austin et al., 1995; McCabe et al., 1999). In situ hybridization revealed a pattern of *Shh* mRNA expression as a center-to-periphery spreading gradient in the inner portion of the retina (Fig. 1A). At stage 24 (embryonic day 4, E4), *Shh* transcripts were detected in sparsely distributed cells occupying the inner surface of the central retina, where newly differentiated ganglion cells resided (Fig. 1C). The expression levels of Shh in these retinal cells were relatively low when compared with Shh hybridization signals in the ventral forebrain on the same sections (data not shown). However, both the intensity of the hybridization signals and the number of Shh-positive cells increased and propagated to more peripheral regions of the retina from stage 24 (E4) to stage 29 (E6) (Fig. 1C,E,G). These expression patterns of *Shh* temporally and spatially correlated with the progression of chick RGC differentiation (Spence and Robson, 1989; Snow and Robson, 1994; Prada et al., 1992; McCabe et al., 1999), supporting that differentiated ganglion cells express *Shh*.

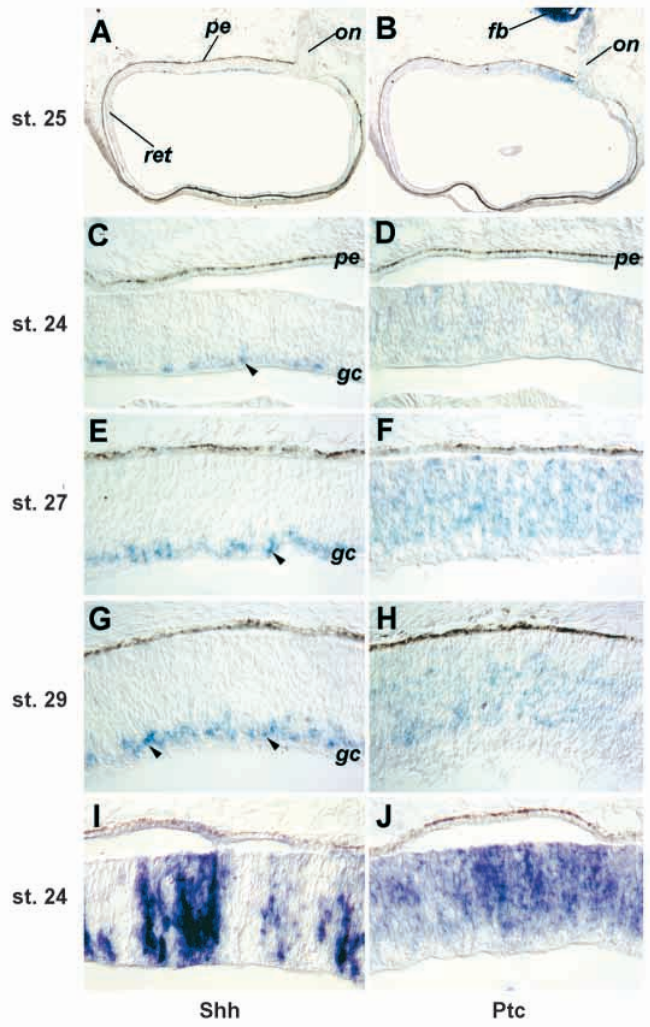

Fig. 1. Expression patterns of Shh and Ptc1 during early chick retinal neurogenesis. In situ hybridization of retinal sections using chick Shh (A,C,E,G,I) and Ptc1 (B,D,F,H,J) probes are shown. Developmental stages according to Hamburger and Hamilton (Hamburger and Hamilton, 1951) are indicated on the left. In adjacent sections of the same eye, Shh is expressed as a gradient in the inner retina (A), whereas Ptc1 shows a similar gradient of expression with the highest levels near the optic nerve head (B). Also note the intense Ptc1 signals in the ventral forebrain, which reflects the significantly higher levels of Shh expression in the vicinity (data not shown). Hybridization signals of Shh are localized in the inner portion of the retina between stage 24 and 29 (arrowheads in C,E,G). No Shh signals are detected in the pigmented epithelium or in the periocular mesenchyme. Ptc1 signals are detected in the proliferative zone between stage 24 to 29 complimentary to the inner retina (D,F,H). Nearby sections of a stage 24 retina infected by the Shh virus at stage 10 (I,J) demonstrate that the non-uniform viral-mediated Shh expression results in a broad induction of Ptc1 mRNA transcription in the ventricular zone of the infected retina. *fb*, forebrain; *gc*, ganglion cells; *on*, optic nerve; *pe*, retinal pigmented epithelium; *ret*, retina.

Between E4 and E6, no *Shh* mRNA was detected by in situ hybridization in the pigmented epithelium layer (Fig. 1C,E,G). These results are consistent with previously described patterns of *Shh* expression in the mouse and zebrafish retinas (Jensen and Wallace, 1997; Wallace and Raff, 1999; Neumann and Nuesslein-Volhard, 2000), suggesting evolutionarily conserved functions of Shh in early retinal neurogenesis.

We also examined expression of the Shh receptor, Ptc1, during early retinal development. Since Shh signaling through its receptors further induces the transcription of Ptc1 in target cells (reviewed by Goodrich and Scott, 1998), the accumulation of Shh signals expressed by RGCs should result in the enhanced expression of *Ptc1* mRNA in the retina. A striking *Ptc1* mRNA expression gradient mirroring the *Shh* expression pattern was observed in the retina (Fig. 1B). The induced Ptc1 gradient corresponded to the RGC differentiation wave spatially and temporally, with the highest level near the optic nerve head, where the wave originates (McCabe et al., 1999; Masai et al., 2000). From stage 24 to stage 27 (E5), increasing levels of *Ptc1* mRNA expression were detected in the retina (Fig. 1D,F). By stage 29, *Ptc1* expression became more restricted to the middle portion of the retina, excluding the ventricular surface and the inner retina (Fig. 1H). Thus, *Ptc1* mRNA is induced in the proliferative zone complementary to the inner retina occupied by differentiated RGCs. These results indicate that as individual RGCs become more mature, they secret Shh signals that affect the adjacent progenitor cell population. As a consequence of the increased numbers of RGCs, higher levels of Shh signal are present further behind the RGC differentiation wave front.

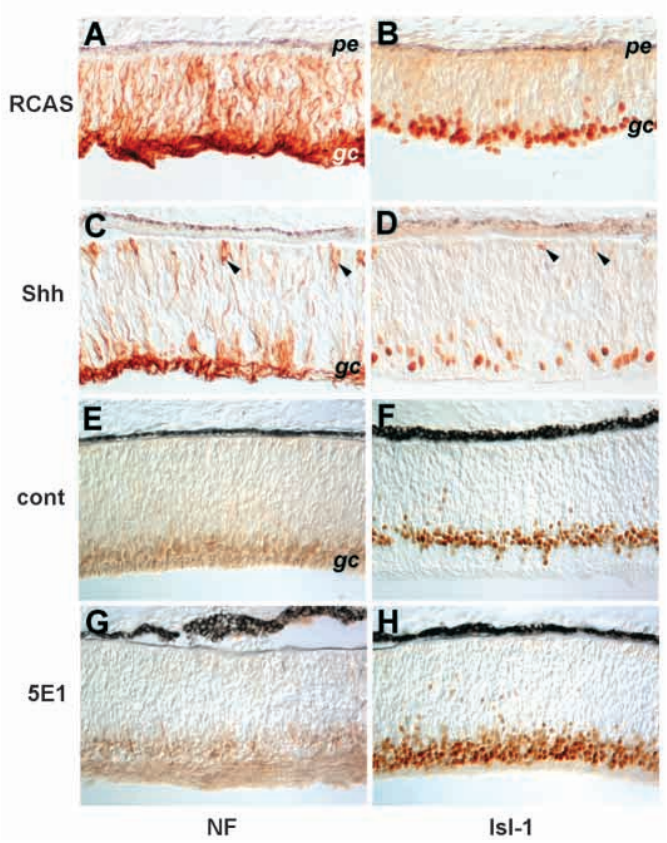

Fig. 2. Effects of perturbing Shh signals on ganglion cell differentiation in vivo. Immunohistochemical staining of central retina sections with anti-neurofilament (NF68) (A,C,E,G) or anti-Islet 1 (Isl-1) (B,D,F,H) antibodies are shown. Compared with the control retina infected by the RCAS virus at stage 10 (A,B), Shh-virus infected retina (C,D) shows a reduction of NF68 and Islet 1-positive cells at stage 24. Shh virus infected retinas also have increased thickness. Arrowheads point to marker-positive cells, possibly differentiating ganglion cells, present near the ventricular surface of Shh virus-infected retina. Compared with retina derived from the control 3C2 hybridoma cell injected eye (E), 5E1 hybridoma cell influenced retinas (G) display a thicker ganglion cell fiber layer stained positive for NF68 at stage 29. In addition, 5E1 hybridoma cell influenced retinas (H) also show increased number of Islet 1-positive cells compared with the non-injected contralateral retina (F) from the same embryo. *gc*, ganglion cell layer; *pe*, pigmented epithelium.

Perturbation of Shh signals in vivo affects ganglion cell production

The expression patterns of *Shh* and *Ptc1* in the chick retina suggested that Shh protein secreted by RGCs in the developing retinal environment might act as a cell-extrinsic factor to influence the early progenitor cell population. It is conceivable that Shh acts as a negative regulator of the chick RGC production (Waid and McLoon, 1998). To test this hypothesis, we examined effects of perturbing endogenous levels of Shh on ganglion cell production in vivo.

As a means to increase the level of Shh signal, we infected the developing chick retinal primordium with replication competent retroviruses expressing the Shh protein (Riddle et al., 1993). The *Shh*-expressing virus or the control RCAS virus were injected into the developing optic vesicles at stage 10 (E1.5, 10 somites) or into the subretinal space of the optic cup between the pigmented epithelium and the retina layers at stage 17-18 (E2.5) prior to the onset of retinal differentiation. The efficacy of Shh viral infection was assessed by in situ hybridization or by immunocytochemical staining. Infection of stage 10 optic vesicles typically resulted in large areas of the retina expressing high levels of Shh at stage 24 (Fig. 1I). A corresponding increase in the *Ptc1* mRNA level was also observed in adjacent retinal sections (Fig. 1J). Although Shh virus infection was often non-uniform, the induction of *Ptc1* mRNA by ectopic Shh was more broadly distributed throughout the retina, suggesting that Shh signals secreted by virally infected cells acted non cell-autonomously to enhance *Ptc1* expression across the retina. However, no obvious *Ptc1* induction was found in the inner retina occupied by differentiated ganglion cells, indicating that differentiated RGCs themselves maybe refractory to Shh signals as found in non-infected retina at these stages. Similar high efficiency of viral infection was observed for stage 17 optic cup injection using anti-viral protein antibody 3C2 (data not shown).

The influence of viral-mediated Shh misexpression on RGC differentiation was examined by immunocytochemistry using cell type-specific markers. In chick, presumptive RGCs express neurofilament proteins either within, or as soon as they exit the metaphase of their last mitotic cell cycle (Waid and McLoon, 1995; McCabe et al., 1999), and neurofilament proteins remain RGC-specific throughout retinal neurogenesis (Torelli et al., 1989; Jasoni et al., 1994; Austin et al., 1995). In stage 24 control retinas, anti-neurofilament antibody (NF68) strongly stained the ganglion cell layer as well as newly postmitotic

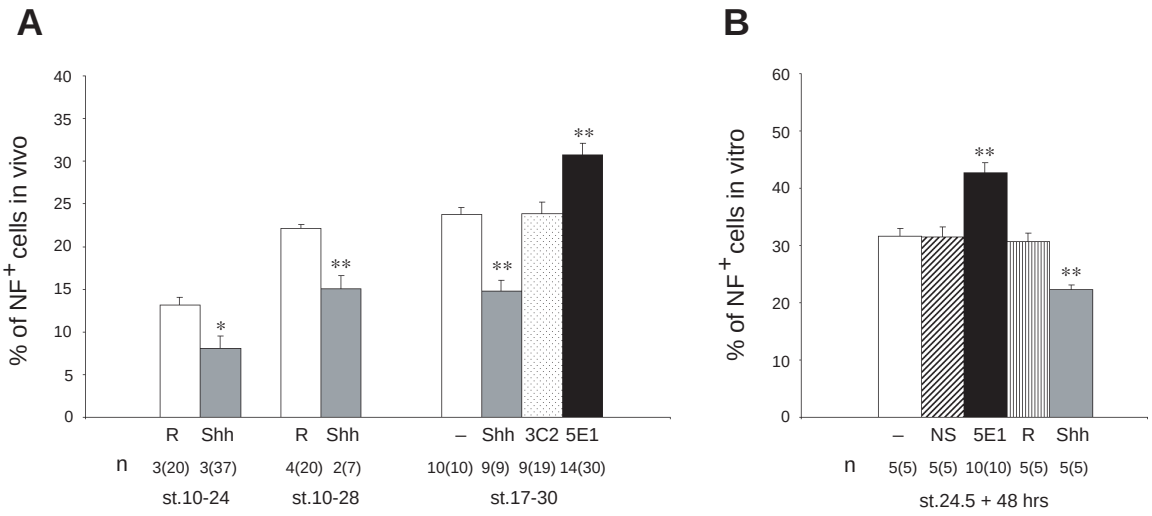

Fig. 3. Quantification of ganglion cell production under different Shh levels in vivo and in vitro. Histograms of percentages of NF68 positive cells among total cells are shown. In this figure and subsequent figures (Figs 6, 8), each bar represents mean±s.e.m. The asterisks * and ** indicate *P* values between 0.01-0.05 and ≤ 0.01, respectively. Numbers outside the parentheses under the horizontal axis represent numbers of individual trials conducted; numbers within the parentheses indicate the total number of retinas used. (A) In vivo infection at stage 10 or stage 17 with Shh virus results in reduction of NF68-positive cells at stages 24, 28 and 30 compared with control RCAS virus (R) infection. In vivo injection at stage 17 with 5E1 hybridoma cells leads to an increase of NF68-positive cells at stage 30, whereas injection of control 3C2 hybridoma cells has no effects. (B) Retinal explants (center 75%) established at stage 24.5 and cultured under different conditions in vitro for 48 hours contain different proportions of NF68-positive cells. Viral-mediated Shh expression leads to a decrease of NF68-positive cells, whereas addition of 5E1 antibody results in an increase of NF68-positive cells. Exposure to control RCAS virus (R) or to hybridoma culture medium (NS) show the same percentages of marker positive cells as non-treated explants (–).

ganglion cells that were leaving the ventricular surface en route to the inner retina (Fig. 2A). Shh virus-infected retinas showed significantly reduced NF68 immunostaining signals compared with control retinas, both in the inner retina occupied by differentiated ganglion cells and across the entire retina (Fig. 2C). Moreover, the distribution of NF68-positive cells was restricted to the ventricular surface and the inner retina with sparsely distributed processes in the middle portion of the retina. Immunocytochemical analyses of the LIM-domain-containing protein Islet 1, which is expressed in differentiated ganglion cells at early stages of chick retinal development (Austin et al., 1995; Yamada et al., 1993), revealed similar abnormalities in RGC development. Control retinas displayed low levels of Islet 1 staining in the proliferative zone and intense nuclear staining for Islet 1 in 2-3 rows of cells within the inner layer of the retina (Fig. 2B). Some Islet 1-positive cells were also detected in the middle of the control retina, suggesting that Islet 1 expression was upregulated in differentiating ganglion cells located near their final destination. The Shh virus-infected retina displayed reduced staining of Islet 1 in the entire retina with fewer Islet 1-positive cells showing intense staining signals (Fig. 2D). Furthermore, some Islet 1-positive cells were ectopically located near the ventricular surface. These results show that misexpression of Shh in the retina caused reduced ganglion cell production in vivo during the period of early neurogenesis.

Table 1. Effects of Shh on cell differentiation and proliferation in vitro

Treatment	DIV0 (E4)	DIV2 (E6)	DIV3 (E7)	DIV4 (E8)	DIV5 (E9)
	% NF^+ cells				
No addition	20.9±1.58	–	–	–	–
RCAS virus		32.4±1.1	18.3±1.2	15.5±0.9	15.5±2.3
Shh virus		24.8±2.3*	13.2±1.0*	10.6±1.2*	12.5±1.2
3C2 antibody		30.3±2.9	19.0±0.8	17.3±1.6	16.9±4.1
5E1 antibody		36.9±1.5*	20.6±2.0	17.9±1.1	14.7±1.1
	% $BrdU^+$ cells				
No addition	43.9±1.8	–	–	–	–
RCAS virus		22.8±1.4	14.0±0.8	7.1±0.5	4.0±0.9
Shh virus		19.3±1.3	15.7±1.0	7.5±0.6	2.6±0.4
3C2 antibody		22.2±1.8	16.2±1.5	7.1±0.6	2.7±0.2
5E1 antibody		24.1±1.2	13.9±1.0	7.3±0.4	3.2±0.4

Retinal explant cultures were established at E3.5 (HH stage 22) and cultured for 12 hours before addition of antibodies and viruses. At this point (DIV0), some explants were dissociated and percentages of marker-positive cells were determined. At DIV3 (E7), cultures were supplemented with 1 ml of fresh medium with or without antibodies according to original conditions. BrdU (20 μM) was added to the medium 3 hours before dissociation of explants at designated times. Data represent four separate explant cultures (n=4) with total of eight retinas for each condition. Percentages of marker positive cells among total cell populations are shown as mean±s.e.m. *P=0.01-0.05.

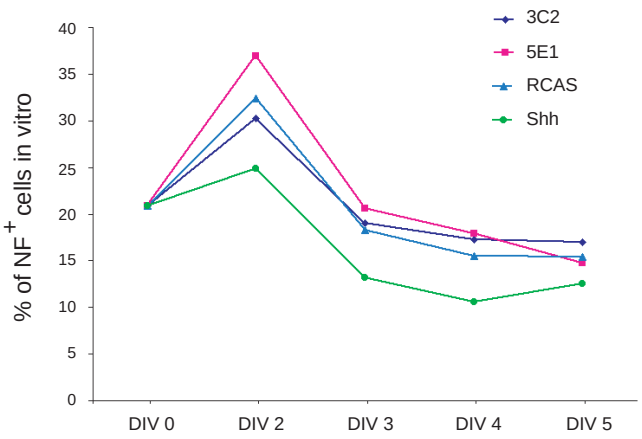

Fig. 4. Influence of Shh levels on temporal differentiation of ganglion cells in vitro. Percentages of NF68-positive cells among total cells in retinal explants cultured for 5 days in vitro (DIV) are shown. Retinal explants were established at stage 22 and cultured under the same condition for 12 hours before the DIV0 samples were assayed. Different types of treatments, including infection with Shh virus or control RCAS virus and exposure to the anti-Shh 5E1antibody or the control 3C2 antibody, were initiated at DIV0. After 48 hours (DIV2), explants were sampled at every 24 hours till DIV5. The peak times for RGC production were the same (DIV2), despite different percentages of RGCs under various Shh conditions. Percentages of RGCs show gradual decline during later culture periods. Shh virus infected explants continued to show statistically significant lower percentages of RGCs at DIV3 and DIV4 compared with controls. Detailed data are summarized in Table 1.

In order to block endogenous Shh activity in vivo, a Shh-neutralizing monoclonal antibody 5E1 (Ericson et al., 1996) was used. The anti-Shh antibody producing hybridoma cells (5E1, IgG) and control hybridoma cells (3C2, IgG) producing an anti-viral antibody (Stoker and Bissell, 1987) were injected into the vitreal space of stage 17 or 18 (E2.5) eyes. Immunocytochemical staining of neurofilaments at stage 29 (E6) showed thickened ganglion cell fiber layers in 5E1 antibody treated retinas (Fig. 2G) compared with the control retina (Fig. 2E). Whole-mount staining of retinas for neurofilament markers also revealed aberrant axonal trajectories of ganglion cells in 5E1 antibody treated eyes (data not shown). Consistent with neurofilament marker expression, an increased number of Islet 1-positive cells was detected in the inner portion of retinas derived from the 5E1 cell injected eyes (Fig. 2F,H). Thus, reduction of endogenous Shh appeared to enhance RGC production.

To further confirm these observed effects of in vivo perturbation, we performed quantitative marker analyses. Infection with Shh-expressing virus either at the optic vesicle (stage 10) or the optic cup (stage 17) stages resulted in a 30-40% reduction of neurofilament positive cells from the control levels between stage 24 and stage 30 (Fig. 3A). Conversely, injection of 5E1 hybridoma cells into the optic cup caused approximately 20% increase of neurofilament-positive cells from the level found in control hybridoma injected eyes (Fig. 3A). Together, these data suggest that endogenous Shh signals negatively regulate the production of RGCs during the period of ganglion cell genesis.

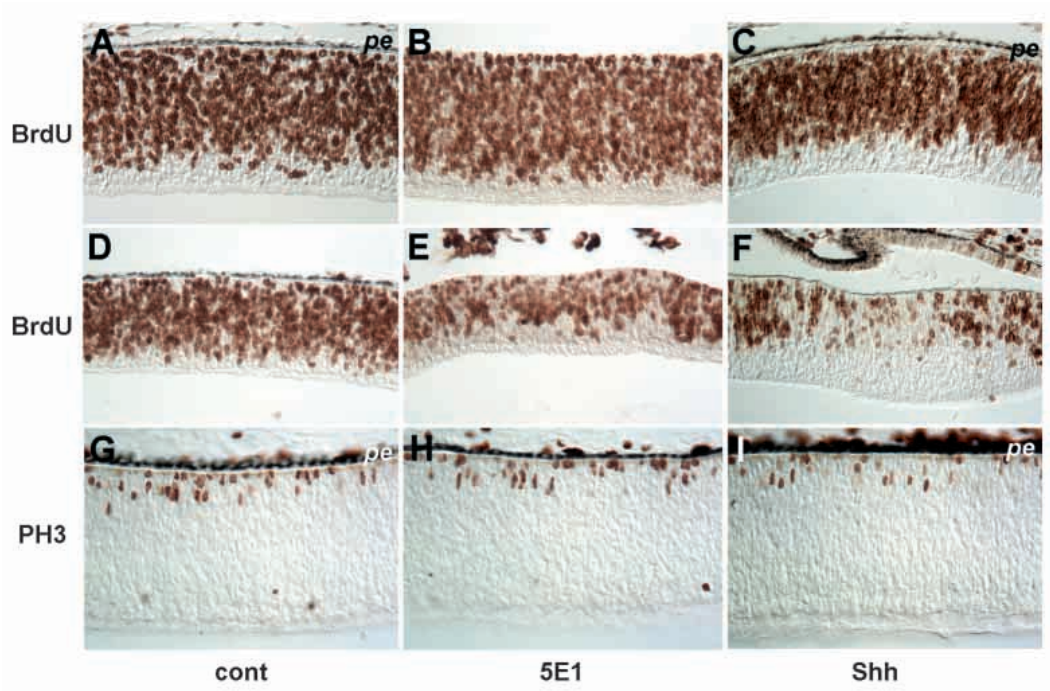

Fig. 5. Effects of Shh on cell proliferation in vivo. Immunohistochemical staining of E6 retinas treated with Shh virus and anti-Shh 5E1 hybridoma cells at stage 17 are shown. Central (A-C) and ventral (D-F) regions of retinas labeled by BrdU for 6 hours in vivo are stained by the anti-BrdU antibody. No effect of perturbing Shh on BrdU incorporation is found in the central retina; however, both elevated and reduced Shh levels result in less BrdU incorporation in the ventral retina near the optic fissure. Central regions of retina stained for the anti-phospho-histone H3 (PH3) show similar patterns of PH3-positive cell distribution (G-I). No differences are detected among non-treated controls, 3C2 hybridoma cell injected, and RCAS virus infected eyes (data not shown). *pe*, pigmented epithelium.

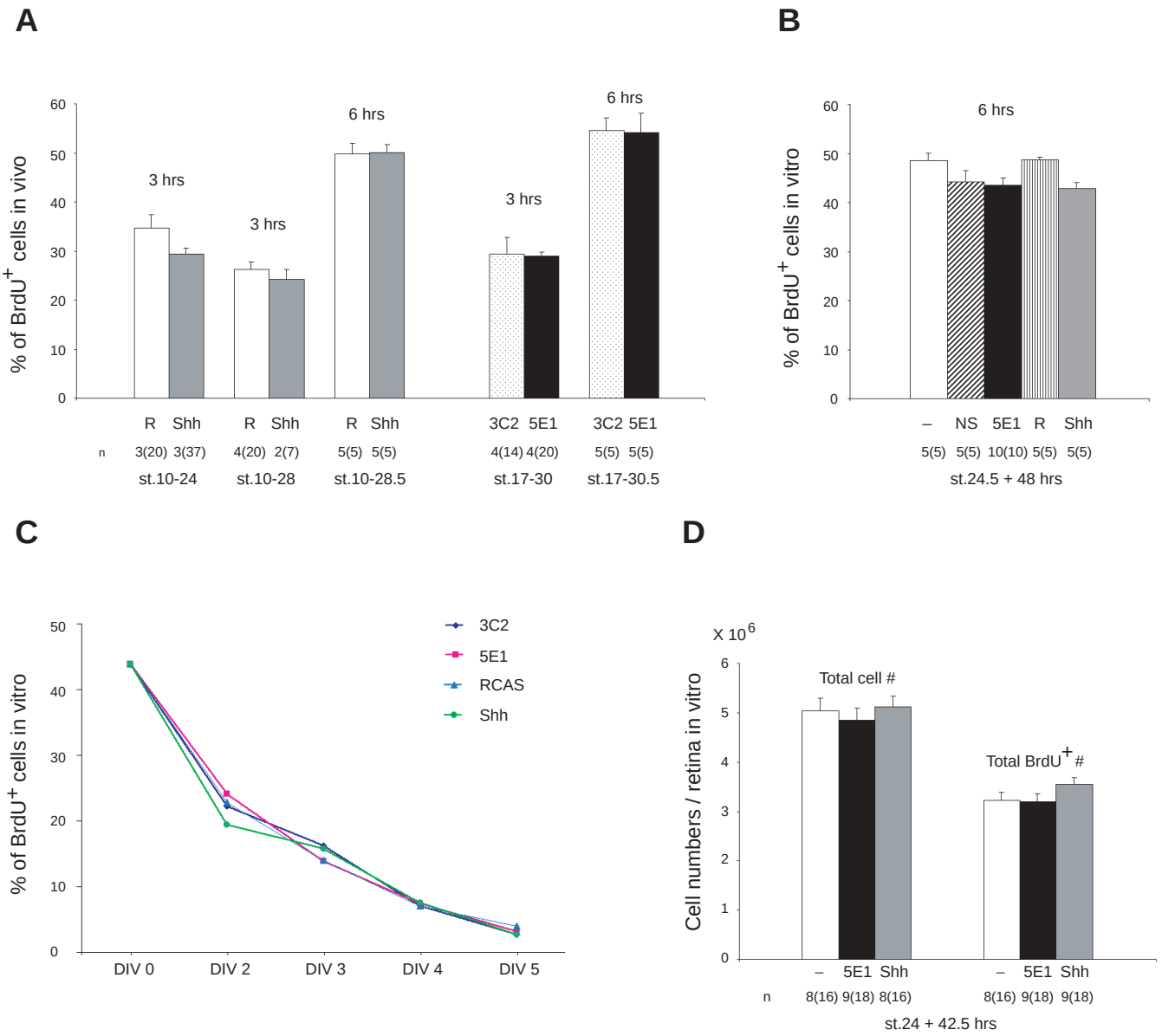

Fig. 6. Quantification of cell proliferation and cell numbers in vivo and in vitro. Histograms of percentage of BrdU-positive cells (A-C) and total cell numbers (D) are shown. (A) BrdU labeling was performed in ovo for 3 hours or in vitro for 6 hours using freshly dissected retinas. For retinas infected at stage 10, Shh virus-infected and control RCAS virus (R)-infected retinas show similar percentages of BrdU-positive cells at stage 24 and stage 28. For eyes injected with hybridoma cells at stage 17, anti-Shh 5E1 cell and control 3C2 cell treat retinas display identical levels of BrdU incorporation at stage 30. (B) Retinal explants (center 75%) were established at stage 24.5 and exposed to different Shh conditions for 48 hours with BrdU added for the last 6 hours of culture. Similar levels of BrdU incorporation were found in anti-Shh 5E1 antibody- or Shh virus-treated retinas as in control retinas exposed to the hybridoma medium (NS), the RCAS virus (R), as well as the non-treated retinas (–). (C) Retinal explants were established at stage 22 and cultured in vitro for 12 hours before the DIV0 samples were assayed. Different treatments, including viral infection and antibody addition, were initiated at DIV0. After 48 hours (DIV2), explants were sampled at every 24 hours until DIV5. Explants were cultured in the presence of BrdU for the last 3 hours before dissociation and staining. No statistically significant changes of BrdU incorporation were detected in Shh virus infected or 5E1 antibody treated explants compared to RCAS virus infected or 3C2 antibody treated controls. See Table 1 for details. (D) Whole retinas were cultured as explants at stage 24 for 42.5 hours in vitro. Prior to dissociation at the end of the culture period, retinas were incubated in the presence of BrdU for 12 hours. Total cell numbers were determined based on cell counting. Total numbers of BrdU marker-positive cells were calculated based on percentages of BrdU-positive cells. Control retinas (–), 5E1 antibody-treated and Shh virus-infected retinas display similar numbers of total cells per retina as well as similar numbers of BrdU-labeled cells per retina.

Shh signals play a regulatory role on ganglion cell genesis in retinal explants

Perturbation of Shh signals in chick during optic vesicle to optic cup transition period affects pattern formation of the eye primordium (Zhang and Yang, 2001). In order to reduce or eliminate the patterning effects of Shh and influences asserted

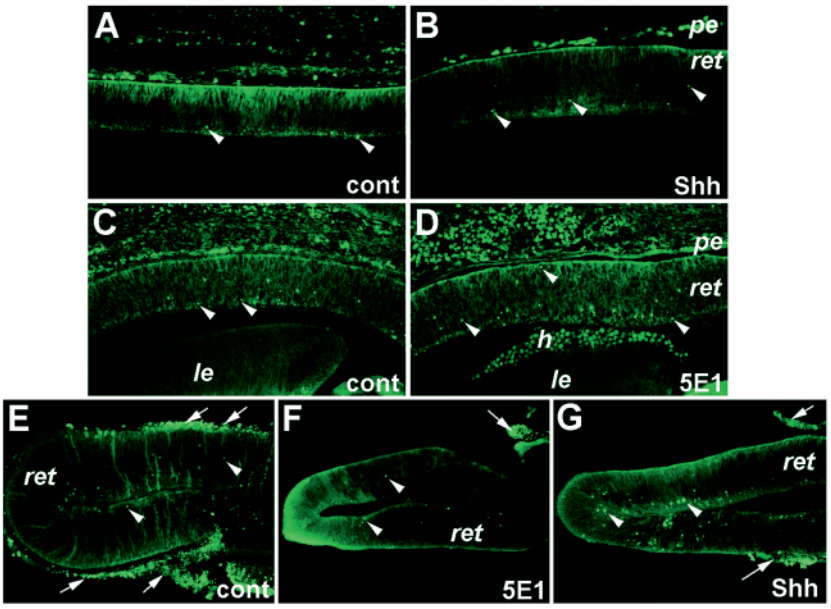

Fig. 7. Effects of Shh on cell death. TUNEL staining of eye sections treated in vivo (A-D) and retinal explant sections treated in vitro (E-G) are shown. Arrowheads point to a subset of TUNEL-positive cells. The right eyes were infected with the Shh virus (B) or injected with 5E1 hybridoma cells (D) at stage 17, and the corresponding left eyes (A,C) of the same embryos are used as stage matched controls. Similar regions of the two retinas show that the Shh virus-infected (B) retina has similar level of cell death at stage 30 (E6) as the non-infected left retina (A). The viral infection was limited to the right eye as confirmed with anti-viral GAG antibody staining (data not shown). Similar regions of two retinas show that the 5E1 cell influenced retina (D) contained slightly more apoptotic cells at stage 27 (E5) than the non-injected left retina (C). Control 3C2 hybridoma cell injected eyes showed similar levels of cell death as the non-treated retinas (data not shown). Representative sections of Stage 24 retinal explants cultured for 48 hours in vitro in the presence of 5E1 antibodies (F), Shh virus (G) or without treatment (E, cont) show similar levels of apoptosis. Arrows point to pigmented epithelium. *h*, hybridoma cells; *le*, lens; *pe*, pigmented epithelium; *ret*, retina.

by other ocular tissues such as the pigmented epithelium and the optic stalk, we next altered Shh signal levels in the neural retina itself within the peak period of chick RGC production (between E4 and E7), when initial eye morphogenesis was complete. At stage 24 (E4), the differentiation wave front of RGC has spread across approximately 75% of the central retina; however, the area behind the RGC wave front continues to give birth to ganglion cells. When the center 75% of the stage 24 retinas were cultured as explants in vitro and subjected to distinct treatments that perturbed Shh levels, changes in the proportion of differentiated RGC cells among the total cell population were detected within 48 hours (Fig. 3B). The non-infected and control virus-infected retinal explants displayed similar levels of RGCs (30% NF68-positive cells); Shh virus infected explants showed a 20-25% reduction of NF68-positive cells compared with controls. In contrast, 5E1 antibody-treated retinas showed a 25% increase in RGCs compared with control retinas. These results indicate that Shh signals produced in the neural retina play a regulatory role in controlling RGC production during the neurogenic phase of eye development. This function of Shh is distinct from the role of ventral midline derived Shh in eye morphogenesis and patterning.

Altering Shh signals does not shift the period of ganglion cell birth

One possible mechanism by which altered levels of Shh signal might influence RGC production is to influence the peak period of ganglion cell birth, thus resulting in changes in the proportion of RGCs at a given developmental stage. To investigate this possibility, we examined the effects of Shh levels on RGC production in retinal explants over a period of 5 days in vitro. Control stage 24 retinal explants showed the highest proportion of RGCs among total cells after two days in vitro (DIV2), corresponding to E6 in vivo (Fig. 4). Thereafter, the percentages of RGCs declined in control cultures, which was likely to be due to continued cell proliferation and generation of other retinal cell types in the culture. Both Shh virus and 5E1 antibody treated retinas showed statistically significant decrease or increase in the percentages of RGCs, respectively, at DIV2; however, the peak production time of RGCs (DIV2) was not affected by the different Shh environments (Fig. 4; Table 1). The Shh virus treated retinas exhibited lower percentages of RGCs during the remainder of the culture period (Fig. 4; Table 1). In contrast, the 5E1 antibody treated retinas did not maintain a statistically significant increase in RGCs after DIV2 (Fig. 4; Table 1). This lack of effect of 5E1 on RGC in later cultures may be due to the depletion of the 5E1 antibody in culture; alternatively, it may reflect cumulative changes of retinal cells to reduced Shh signals. These data suggest that instead of shifting the timecourse of RGC development, Shh signals act within the normal competent period of progenitor cells for RGC specification (Austin et al., 1995; Waid and McLoon, 1998) to affect RGC genesis.

Effects of Shh on retinal cell proliferation and death

The complimentary expression patterns of *Shh* and its receptor Ptc1 in the retina suggest that secreted Shh molecules act in a paracrine fashion to influence progenitor cell behaviors. We, thus, examined if altering Shh levels affected progenitor cell proliferation and/or retinal cell death, and consequently changed the proportion of RGCs.

First, we determined if Shh acted as a mitogen in vivo at stage 17 after the optic cup was formed. Immunostaining of BrdU-positive cells at stage 30 (E6.5) showed that after 6 hours of in vivo labeling, marker-positive cells were distributed throughout the retina excluding the RGC layer (Fig. 5A). No detectable differences in BrdU labeling patterns were observed in the central regions of control, 5E1 cell, or Shh-treated retinas (Fig. 5A-C). However, under both elevated and reduced Shh conditions, regions flanking the ventral optic fissure showed a reduction of BrdU incorporation (Fig. 5D-F), reflecting the

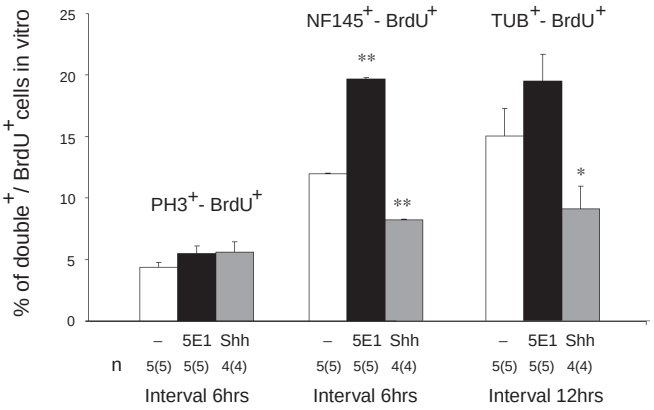

Fig. 8. Shh levels affect ganglion cell fate specification in vitro. Histograms show percentages of double marker-positive cells among BrdU-positive cells. Retinal explants (center 75%) were established at stage 23.5 and cultured for 40 hours under conditions without any treatment (–), or exposed to Shh viruses or 5E1 antibodies from the beginning of the culture period. The explants were then labeled with BrdU for 2.5 hours, followed by washes and further incubation in fresh medium for either 6 or 12 hours. At 6 hours after the BrdU labeling, retinal explants show similar percentages of phosphohistone H3 (PH3, a M phase marker) and BrdU double-positive cells among all BrdU-labeled cells. However, at 6 hours post BrdU labeling, Shh virus-infected and 5E1 antibody-treated explants display decreased and increased percentages of neurofilament (NF145) and BrdU double-positive cells among all BrdU-labeled cells, respectively. By 12 hours post BrdU labeling, β-tubulin (TUB) and BrdU double-positive cells emerged. Shh viral infection causes a decrease, and 5E1 antibody treatment causes an increase of β-tubulin and BrdU double-positive population among BrdU-positive cells.

patterning effect of Shh signals on eye morphogenesis (Zhang and Yang, 2001). Similarly, staining with the anti-phosphohistone H3 (PH3) antibody, which labels cells in the late G2 phase and metaphase (Wei and Allis, 1998; McCabe et al., 1999; Masai et al., 2000), did not reveal qualitative differences among control and manipulated retinas (Fig. 5G-I). Consistent with immunostaining patterns for progenitor cell markers, quantitative analyses of retinas in elevated or reduced Shh environments in vivo also showed no significant changes of BrdU incorporation (Fig. 6A).

Next, we examined if Shh signals had mitogenic activities in vitro during the period of RGC genesis, using retinal explants established and subjected to different treatments at stage 24 (E4). The center regions (75%) of the retina cultured under distinct Shh environments for 2 days showed no significant difference in the percentages of BrdU-positive cells after 6 hours labeling (Fig. 6B). Prolonged explant cultures for up to 5 days in vitro (corresponding to E4 to E9 in vivo) displayed a gradual decline of cell proliferation; however, similar levels of BrdU incorporation were found at all stages under either elevated or reduced Shh conditions (Fig. 6C; Table 1). To further evaluate if Shh signal levels affected total cell numbers and therefore percentages of marker positive cells, whole retinal explants including both center and peripheral portions were established. At stage 24 (E4) each retina contained on average $0.9(\pm 0.17)\times 10^6$ cells. After 42.5 hours of in vitro culture, the control explants contained on average 5×10^6 cells/per retina, while retinas cultured under elevated or reduced Shh conditions contained similar numbers of cells (Fig. 6D). Moreover, total numbers of BrdU-labeled cells per retina were not affected by Shh levels after a 12 hour labeling period in these whole retinal cultures (Fig. 6D).

Since the effects of Shh on ganglion cell production did not correlate with altered cell proliferation, we investigated if manipulating Shh levels affected retinal cell death. In normal stage 24 to 27 (E4 to E5) chick retinas, TUNEL assay revealed small numbers of apoptotic cells that were typically located in the ventral retina near the optic nerve head (Zhang and Yang, 2001). Infection of retina by the Shh virus at stage 17 during eye morphogenesis resulted in a significant increase of cell death in the ventral retina prior to E5 accompanied by the loss of ventral retina tissues (data not shown). However, by E6, the remaining Shh virus infected retinal tissues showed similar levels of apoptosis compared to control retinas (Fig. 7A,B). Injection of 5E1 hybridoma cells at stage 17 caused a slight increase of apoptosis by E5 (Fig. 7C,D). These results suggest that although aberrant Shh levels in the newly formed optic cup could critically affect apoptosis in the retina, these effects of Shh diminished as the retina entered the neurogenic period. To further evaluate if Shh signals affected apoptosis during ganglion cell genesis in vitro, stage 24 explants cultured in vitro were sectioned and assayed by TUNEL. Despite an overall increase of apoptosis in vitro, explants cultured under elevated or decreased Shh conditions did not display significantly different levels of apoptosis (Fig. 7E-G). Since the dying cells labeled by the TUNEL assay were not well labeled by other cell markers and were presumably quickly removed, the identities of these dying cells in either Shh virus or 5E1 antibody treated retinas could not be determined unambiguously.

Together, the in vivo cell proliferation and cell death data suggest that Shh does not serve as a mitogen during early retinal development, and that the effects of Shh on apoptosis diminishes when the retina reaches the neurogenic phase of development. The lack of Shh effects on total cell numbers, cell proliferation and cell death in vitro during the peak period of RGC production further supports its role in cell differentiation.

Shh signals influence the specification of ganglion cells

In other regions of the developing central nervous system, graded Shh signals affect cell fate specification of neuronal progenitor cells (Ericson et al., 1997; Dahmane and Ruiz i Altaba, 1999; Ye et al., 1998). Since Shh levels did not significantly affect cell death or proliferation in vitro during RGC genesis, we next examined if Shh signaling influenced cell fate choices of early retinal progenitor cells. Retinas cultured under different Shh environments for 40 hours were exposed to BrdU for 2.5 hours in order to label S phase cells. Subsequently, the cohort of BrdU-positive progenitor cells were monitored for their progression into the M phase of the cell cycle by the phosphohistone H3 marker (PH3), and for their specification into the ganglion cell fate by neurofilaments (NF) and β-tubulin expression. At 4, 6 and 8 hours after BrdU labeling, control retinas showed identical levels (4.5-5.5%) of PH3 and BrdU double-positive cells, indicating that the BrdU-labeled cohort of cells was steadily progressing through the M phase at maximal levels between 4 and 8 hours post S phase.

At 6 hours post-labeling, Shh virus-infected and 5E1 antibody-treated retinas showed similar percentages of PH3 and BrdU double-positive cells as controls (Fig. 8). Thus, Shh levels did not appear to disrupt progenitor cells progression from the S phase to the M phase of the cell cycle.

Since progenitor cells adopting the ganglion cell fate began to express neurofilament proteins during or soon after their terminal M phase at the ventricular zone, neurofilaments were used to monitor the onset of ganglion cell fate specification. At 6 hours post BrdU labeling, 12% of BrdU-labeled cells also stained positively for neurofilament proteins (NF145) in the control explants (Fig. 8). Significantly, 5E1 antibody-treated retinas showed an increase of NF145 positive cells (18%) among BrdU labeled cells; conversely, Shh virus infected retina showed a decrease of NF145 and BrdU double positive cells (8%) (Fig. 8). These changes are not due to varied BrdU incorporation under distinct Shh environments, because identical proportions of BrdU positive cells (40%) were found under all Shh conditions. Therefore, Shh signaling affected the ability of progenitor cells to initiate the differentiation program towards becoming ganglion cells.

In contrast to neurofilaments, β-tubulin-positive cells are restricted to the inner surface of the retina between E4 and E6 (data not shown), indicating that β-tubulin is expressed only by more mature RGCs in their final location. Consistent with this, very few BrdU and β-tubulin double-positive cells were detected in retinal explants 8 hours after BrdU labeling; however, by 12 hours, a BrdU and β-tubulin double-positive population emerged. Compared with the control level of 15% β-tubulin and BrdU double-positive cells, 5E1 antibody treatment increased the double marker positive RGC population to 20%, whereas Shh virus infection decreased this population to 10% (Fig. 8).

Together, these results suggest that Shh signaling influences the progenitor cell fate decision during M phase of the last mitotic cell division, and/or the subsequent differentiation of nascent ganglion cells.

DISCUSSION

The Hedgehog family of proteins has been shown to play multiple roles in the development of invertebrate and vertebrate visual systems. Results presented here reveal a novel function of *Shh* in regulating vertebrate retinal ganglion cell genesis. We demonstrate that during the neurogenic phase of chick retinal development, Shh signals derived from differentiated RGCs act in a paracrine fashion to influence uncommitted early progenitor cells. Elevating Shh signals leads to decreased production of RGCs, whereas reducing Shh signals results in enhanced RGC genesis, both in vivo and in vitro. Shh signals modulate ganglion cell production within the normal period of RGC genesis without significantly affecting cell proliferation and death in vitro. Furthermore, the effects of Shh signaling on RGC fate specification occur during or soon after the last mitotic cycle of progenitor cells. These findings support our hypothesis that Shh secreted by RGCs serves as a negative regulator of ganglion cell production behind the early neurogenic wave front.

The specification of diverse vertebrate retinal cell types is thought to be, in part, influenced by the changing retinal environment, due to the accumulation of differentiated cells that produce inductive and/or inhibitory signals (Cepko et al., 1996; Harris, 1997; Cepko, 1999). In the mature retina, several cell types occupying distinct laminar layers, including photoreceptor cells, amacrine cells and ganglion cells, show organized patterns (Dowling, 1987; Cepko, 1996; Masland and Raviola, 2000). Molecular mechanisms controlling the density and distribution patterns of distinct retinal cell types are not well understood. However, previous studies have provided supporting evidence for the existence of feedback regulation in cell type determination and patterning. For example, differentiation of retinal inter neurons, the amacrine cells, is affected by an inhibitor produced by the amacrine cell themselves (Belliveau and Cepko, 1999). In particular, the differentiation of dopaminergic amacrine cells in the retina appears to be negatively influenced by an increased presence of this cell type in the developing retina (Reh and Tully, 1986). In addition, retinal culture studies by Waid and McLoon have identified two fractions of media conditioned by older retinas that inhibit ganglion cell differentiation in younger retinas (stage 24) competent to give rise to ganglion cells (Waid and McLoon, 1998). One fraction has a molecular weight limit of less than 3 kDa, while the second fraction contains inhibitory molecules with molecular weights higher than 10 kDa. Waid and McLoon have further determined that the source of inhibitory activities is the ganglion cells themselves, since depletion of ganglion cells resulted in the loss of inhibitory activities (Waid and McLoon, 1998). Our results suggest that in the early retina, ganglion cell derived Shh can act as a

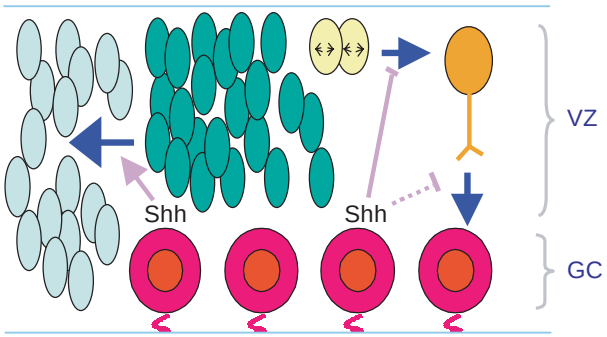

Fig. 9. Proposed dual roles for Shh during early retinal neurogenesis. A schematic cartoon depicts the early retina after RGC differentiation has begun. Shh is secreted by differentiated RGCs (red) located in the inner retina. Ahead of the RGC differentiation wave front naïve retinal progenitor cells (gray) are exposed to low levels of Shh signals, because they are farther away from the Shh-expressing cells. Low levels of Shh signal may be necessary to induce naïve progenitor cells to become competent for differentiation, and some eventually become Shh-producing RGCs. Behind the RGC differentiation wave front, progenitor cells (dark green) residing in the ventricular zone (VZ) are likely to have entered a competent state to be specified as RGCs and may contain activated MAPK. Higher levels of Shh are present behind the RGC wave front due to the accumulation of differentiated RGCs. Shh signaling negatively affects RGC fate specification of competent progenitor cells during or soon after M phase (yellow) of the mitotic cycle, and/or influence the further differentiation of nascent RGCs (orange) migrating towards the ganglion cell layer (GC). This model is consistent with data reported by Neumann and Nuesslein-Volhard (Neumann and Nuesslein-Volhard, 2000).

negative regulator of RGC production. The chick Shh-N has a molecular weight of 19 kDa, and might thus be one of the secreted inhibitory factors that participates in the feedback control of RGC genesis in the retina.

In chick, postmitotic ganglion cells serve as the main source of endogenous Shh mRNA at the initial stages of retinal neurogenesis, since no signals of Shh and other members of the hedgehog family (Desert hedgehog and Indian hedgehog, data not shown) are detected under the same condition by in situ hybridization in the pigmented epithelium between E4 and E6. Judging by the intensity of mRNA signals, RGCs express *Shh* at a considerably lower level than Shh-producing cells located in the ventral forebrain. However, this ambient level of Shh in the early retina appears sufficient to signal progenitor cells in the adjacent proliferative zone, as indicated by the complimentary *Ptc1* expression accumulating behind the differentiation wave front. In the mouse retina at comparable stages of development, similar patterns of *Shh* and *Ptc* expression have been reported (Jensen and Wallace, 1997), and in the zebrafish retina, both *shh* and *twhh* are expressed in ganglion cells (Neumann and Nuesslein-Volhard, 2000). These studies together with our results support an evolutionarily conserved role for Shh signaling in vertebrate retinal neurogenesis. The expression patterns of *Shh* and *Ptc1* also indicate that the Shh signals secreted by ganglion cells can diffuse and influence the progenitor cell population through a paracrine mode of action. However, differentiated RGCs themselves are not responsive to either endogenous or ectopic Shh during early neurogenesis, as indicated by the lack of *Ptc1* expression. At present, it remains unclear how differentiated RGCs activate *Shh* gene transcription and simultaneously gain independence from Shh signals.

Previous studies have shown that Shh has mitogenic activities on certain cell and tissue types (reviewed in Goodrich and Scott, 1998; Wechsler-Reya and Scott, 1999; Dahmane and Ruiz i Altaba, 1999). In the retina, addition of Shh-N protein to mouse E16-P1 retinal cell pellets and monolayer cultures, or to rat E18 monolayer cultures results in enhanced progenitor cell proliferation and overproduction of all late-born cell types (Jensen and Wallace, 1997; Levine et al., 1997). Furthermore, Shh signals secreted by ganglion cell axons promote astrocyte proliferation in the optic nerve in vivo (Wallace and Raff, 1999). Thus, Shh molecules produced by the retina may serve as a mitogen to promote late neuronal and glial progenitor proliferation. We have evidence that in chick, early perturbation of Shh in vivo causes defective eye pattern formation, which is accompanied by increased cell death and decreased cell proliferation in the ventral retina (Zhang and Yang, 2001). The rapid removal of dead cells from the early optic primordium could explain why the percentage of BrdU-positive cells was not affected, despite an observed decrease in total cell numbers in vivo when Shh levels were altered. To eliminate the impact of Shh on eye patterning, we investigated the effects of Shh signaling on cell proliferation and death beyond the morphogenic stages of eye development by using retinal explants. When stage 24 explants were cultured for 42.5 hours, total cell numbers per retina more than quadrupled; however, no effect of distinct Shh environments on cell numbers were observed. In addition, the total number of BrdU-positive cells per retina and consequently the percentages of BrdU-positive cells were not significantly altered by different levels of Shh. Furthermore, retina explants in vitro did not show differential apoptosis when manipulation of Shh signals occurred after stage 24. These data support the idea that secreted Shh does not act as an effective mitogen or affect apoptosis during the period of RGC differentiation in vitro, but instead influences progenitor cell specification.

In the normal retina, presumptive ganglion precursor cells completing mitosis at the ventricular surface immediately extend a neurofilament-containing leading process and migrate toward the inner retina (Waid and McLoon, 1995). The rapid phenotypic differentiation of ganglion cells suggests that their fate may be determined during the last mitotic cell cycle, perhaps within the G2 and/or the M phase. The likely steps that Shh may affect are progenitor fate determination during their last mitotic cell cycle and/or the subsequent differentiation and maturation process of newly specified ganglion cells. By monitoring progenitor cells that have recently gone through the S phase of the cell cycle, we have demonstrated that the proportion of S-phase cells entering the M phase is not affected by Shh signaling. Rather, proportions of BrdU-labeled cells that enter the RGC pathway differ significantly according to Shh signal levels. Reducing endogenous Shh leads to an increase of progenitors initiating the ganglion cell program; conversely, overexpression of Shh causes fewer progenitor cells to adopt the ganglion cell fate. Thus, the first detectable step that Shh signaling appears to influence is the transition from mitosis to the ganglion cell marker-expressing daughter cells. Yet, it is also possible that levels of Shh signal impact upon the ganglion cell differentiation process including the subsequent cell migration and further maturation. The evidence supporting this notion includes the abnormal expression of ganglion cell specific markers, the thickened retinal epithelium, the disrupted organization of the ganglion cell layer and the abnormal trajectory of RGC axons (data not shown). Thus, reduced ganglion cell production caused by Shh overexpression may be due to the combined effects of perturbing cell fate specification and differentiation.

The differentiation and precise patterning of the *Drosophila* compound eye requires a graded signaling of Hh molecules secreted by differentiated photoreceptor cells residing posterior to the MF. Low levels of Hh induce decapentaplegic (Dpp) within and ahead of the MF, which acts at long range from the Hh-secreting cells to promote naïve disc cells to enter the 'pre-proneural' state, represented by expression of bHLH genes including *hairy* and *Extramacrochaetae (Emc)* (Greenwood and Struhl, 1999; Brown et al., 1995). The pre-proneural state is limited to a narrow zone ahead of the MF and is required for progenitor cells to initiate neuronal differentiation. Hh signaling is also necessary for the transition from the pre-proneural state to the 'proneural' state, represented by the expression of the bHLH gene *atonal* (Greenwood and Struhl, 1999; Dominguez, 1999). The expression of *atonal*, which is a determinant of the founding R8 cells (Jarman et al., 1994; White and Jarman, 2000) is complex. Cells that are 5-7 ommatidial rows away from the Hh producing cells express *atonal* in a continuous stripe. However, within distances closer to the differentiated ommatidial clusters, *atonal* expression is gradually reduced to regularly spaced subsets of cells (proneural clusters), and eventually limited to only the R8 cells (Dominguez, 1999). Genetic manipulation of various components of the Hh signaling pathway has demonstrated that

low levels of Hh induce *atonal*, whereas high levels of Hh suppress *atonal* (Dominguez, 1999). Thus, Hh signaling performs dual functions in fly eye neurogenesis, i.e. advancing the MF and regulating the precise spacing of ommatidia. These effects are presumably achieved, at least in part, by the differential regulation of *atonal* expression by Hh at distinct thresholds. Moreover, recent data have indicated that the effect of Hh on *atonal* expression may be mediated by an unidentified secondary signal that activates the Raf-MAPK pathway (Greenwood and Struhl, 1999).

The existence of evolutionarily conserved regulatory mechanisms governing the Drosophila R8 photoreceptor cell and the vertebrate RGC cell production has become increasingly evident. Results from Neumann and Nuesslein-Volhard, and results described in this report further establish that hedgehog signaling plays critical roles in vertebrate retinal neurogenesis (Neumann and Nuesslein-Volhard, 2000). During zebrafish retinal development, a positive regulatory loop exists for Shh at the differentiation wave front, where Shh is necessary and sufficient to induce itself and to promote further expansion of the RGC territory. In addition, the inductive effect of Shh only occurs within a narrow zone of cells adjacent to the progressing wave front, suggesting the existence of a pre-proneural zone similar to that found in the fly eye. Moreover, inhibiting Hedgehog signaling results in the blocking of a propagating activated MAPK wave (Neumann and Nuesslein-Volhard, 2000). By using the center 75% of the retina during the peak period of ganglion cell birth, we have focused our investigation on the role of Shh in further production of RGCs behind the initial neurogenic wave front. Our findings support the hypothesis that local Shh signals play a regulatory role in the emergence of nascent ganglion cells from the progenitor pool. This negative feedback function of Shh signaling behind the differentiation wave front does not contradict with the role of Shh in promoting neurogenesis at the wave front (Neumann and Nuesslein-Volhard, 2000). Instead, we favor a model that progenitor cells respond to different thresholds of Shh signals within the vertebrate retina (Fig. 9). Specifically, ahead of the initial neurogenic wave front, low levels of Shh are likely to be present which may induce the expression of proneural genes such as *atonal* within naïve progenitor cells and render them competent to adopt the RGC pathway. Behind the wave front, higher levels of Shh may suppress proneural gene expression selectively among competent progenitor cells and thus negatively regulate ganglion cell production.

During *Drosophila* compound eye differentiation, a lateral inhibition process involving the Notch pathway also operates in singling out the R8 cells within the proneural clusters (Baker et al., 1996). Expression analyses of the *Enhancer of split complex* (*E(spl)*) (Dominguez, 1999), which are direct targets of Notch signaling, in *Smoothened* mutants indicates that Hh signaling does not affect Notch activation within established proneural clusters. Thus, it is likely that combined lateral inhibition by Notch and regulation by graded Hh signals both contribute to the precise organization of the ommatidial arrays (Dominguez, 1999; Greenwood and Struhl, 1999). During vertebrate retinal development, signals mediated by Notch receptors also play important roles in controlling the number and density of RGCs in vertebrate retina (Dorsky et al., 1995; Austin et al., 1995; Henrique et al., 1997). In our study, cell-cell contacts required by Notch signaling have remained intact, since no dissociated cultures were used. It is therefore possible that the observed effects of Shh signals on ganglion cell production were achieved in the presence of normal Notch signaling. Consistent with this notion, RGC production can be further inhibited by RGC-derived secreted signals when the Notch signaling pathway is disrupted (Waid and McLoon, 1998), supporting the involvement of diffusible factors as well as contact-mediated regulations.

Thus, like Hh molecules in *Drosophila* compound eye development, Shh appears to play dual roles in promoting the neurogenic wave and modulating neuronal production in the vertebrate retina. Future investigations aimed at elucidating the regulatory relationships between Shh signals and the proneural genes, as well as possible secondary signals will advance our understanding of mechanisms that underlie vertebrate retinal neurogenesis.

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Note added in proof

The following paper on RGC development in vertebrate retina was published recently.

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