Temporal and Spatial Effects of Sonic Hedgehog Signaling in Chick Eye Morphogenesis

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Proper dorsal–ventral pattern formation of the optic cup is essential for vertebrate eye morphogenesis and retinotectal topographic mapping. Previous studies have suggested that midline tissue-derived Sonic hedgehog (Shh) molecules play critical roles in establishing the bilateral eye fields and in determining the proximal–distal axis of the eye primordium. Here, we have examined the temporal requirements for Shh during the optic vesicle to optic cup transition and after early optic cup formation in chick embryos. Both misexpressing Shh by virus and blocking Shh activity by antibodies resulted in disruption of ventral ocular tissues. Decreasing endogenous Shh signals unexpectedly revealed a sharp morphological boundary subdividing dorsal and ventral portions of the optic cup. In addition, Shh signals differentially influenced expression patterns of genes involved in ocular tissue specification (*Pax6, Pax2,* and *Otx2*) and dorsal–ventral patterning (*cVax*) within the ventral but not dorsal optic cup. Ectopic Shh suppressed expression of *Bone Morphogenetic Protein 4* (*BMP4*) in the dorsal retina, whereas reducing endogenous Sonic hedgehog activity resulted in a ventral expansion of *BMP4* territory. These results demonstrate that temporal requirements for Shh signals persist after the formation of the optic cup and suggest that the early vertebrate optic primordium may be subdivided into dorsal and ventral compartments. We propose a model in which ventrally derived Shh signals and dorsally restricted BMP4 signals act antagonistically to regulate the growth and specification of the optic primordium.   © 2001 Academic Press

Key Words: eye; chick; dorsal–ventral; Sonic hedgehog; *BMP4; Vax; Pax6; Pax2; Otx2.*

INTRODUCTION

Formation of the vertebrate eye involves coordinated morphogenesis of several embryonic tissues, including the surface ectoderm, the neural crest-derived head mesenchymal cells, and the anterior neural tube (reviewed by Graw, 1996; Grainger, 1992; Jean *et al.,* 1998). During chick neural tube closure, the lateral walls of the forebrain (prosencephalon) evaginate and form the bilateral optic vesicles. The ventral-lateral portion of the optic vesicle subsequently invaginates to form the double-layered optic cup with its inner and outer layers destined to become the neural retina and the pigmented epithelium, respectively. An early event of eye development is the establishment of the anterior–posterior (A-P) and dorsal–ventral (D-V) axes in the optic primordium. The early chick optic cup exhibits a morphological D-V asymmetry indicated by a ventrally localized optic fissure and optic stalk, which is continuous with the

ventral diencephalon and later becomes the optic nerve. The dorsal–ventral polarity persists through later stages of eye development and is critical for the topographic projection of retinal ganglion cell axons to the target field during formation of the retinotectal map (reviewed by O'Leary *et al.,* 1999).

The secreted glycoprotein Sonic hedgehog (Shh), a vertebrate homologue of the *Drosophila* segment polarity gene *Hedgehog,* plays important roles in patterning tissues of the vertebrate embryo including the central nervous system (see reviews by Hammerschmidt *et al.,* 1997; Goodrich and Scott, 1998; McMahon, 2000). Both vertebrate and invertebrate hedgehog proteins undergo an autocleavage to yield an active N-terminal fragment (e.g., Shh-N) covalently linked to a cholesterol molecule (Lee *et al.,* 1994; Bumcrot *et al.,* 1995; Porter *et al.,* 1995; Porter *et al.,* 1996a, b). Shh signals are transmitted through binding to the transmembrane protein Patched1 (Ptc1) (Marigo *et al.,* 1996a; Stone *et al.,* 1996). In the absence of Shh, Ptc1 blocks the signaling activity of the membrane protein Smoothened and suppresses downstream gene expression, including its own

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transcription (Alcedo *et al.,* 1996; van den Heuvel and Ingham, 1996). Binding of Shh to the Ptc1 receptor relieves this inhibition of Smoothened by Ptc1 and consequently upregulates *Ptc1* transcription.

Accumulating evidence indicates that Shh activity is involved in pattern formation of the vertebrate eye (reviewed by Jean *et al.,* 1998; Oliver and Gruss, 1997). Embryonic ablation and transplantation have shown that the anterior axial mesoderm (i.e., the prechordal plate), which expresses *Shh,* is required for the separation and formation of the bilateral eye fields (Li *et al.,* 1997). Targeted disruption of the *Shh* gene in mouse results in embryos with severe anterior neural tube defects and a single fused primitive optic vesicle (Chiang *et al.,* 1996). Similarly, mutations in the human *Shh* gene cause a form of holoprosencephaly (HPE3), which is marked by fusion of the cerebral hemispheres, and in severe cases results in the formation of cyclopic eyes (Belloni *et al.,* 1996; Roessler *et al.,* 1996; Ming *et al.,* 1998). Thus, Shh signals emanating from the rostral midline of the early embryo are necessary for segregation of the bilateral eye fields during embryogenesis. Furthermore, Shh activity has been shown to regulate the expression of *Pax6* and *Pax2* genes which normally demarcate the distal (prospective retina, pigmented epithelium, and lens) and proximal (optic fissure and optic stalk) optic primordium, respectively. Overexpression of *Shh* in early (1–4 cell) zebrafish embryos causes a reduction of the *Pax6* and expansion of the *Pax2* expression domains and leads to malformed eyes (Macdonald *et al.,* 1995; Ekker *et al.,* 1995). Thus, Shh activity influences the pattern formation of the eye in part by regulating expression of pertinent *Pax* genes.

Recent studies have shown that the establishment of distinct D-V properties of the developing retina involves the homeobox-containing *Vax* genes (Schulte *et al.,* 1999; Hallonet *et al.,* 1999; Bertuzzi *et al.,* 1999; Barbieri *et al.,* 1999) and the T-box gene *Tbx5* (Koshiba-Takeuchi *et al.,* 2000). In the developing chick optic vesicle, *cVax* and *Tbx5* are expressed in non-overlapping ventral and dorsal domains. The dorsal expression of the *Tbx5* gene appears to be controlled by bone morphogenetic protein 4 (BMP4), which is normally present in the dorsal optic cup (Koshiba-Takeuchi *et al.,* 2000; Furuta and Hogan, 1998). Misexpression of *Tbx5* results in dorsalization of the ventral retina correlated with the loss of the ventral markers *Pax2* and *cVax* (Koshiba-Takeuchi *et al.,* 2000). Conversely, misexpression of *cVax* causes ventralization, as indicated by the induction of *Pax2* and suppression of *Tbx5* in the dorsal retina (Schulte *et al.,* 1999). Furthermore, targeted disruption of the mouse *Vax1* gene results in ventral eye defects, including incomplete closure of the optic fissure, disrupted retinal axon trajectories, and a defective optic nerve (Hallonet *et al.,* 1999; Bertuzzi *et al.,* 1999). These findings suggest that cVax and Tbx5 are critical cell-intrinsic factors mediating D-V specification of the developing retina.

Although perturbation of Shh activity by targeted gene disruption in mouse (Chiang *et al.,* 1996) and by overex-

pression in early zebrafish embryos (Macdonald *et al.,* 1995; Ekker *et al.,* 1995) has established the important roles of Shh in separation of the bilateral eye fields and in axial pattern formation of the eye, it remains to be determined if Shh activity is necessary during later stages of eye morphogenesis. Expression patterns of *Shh* and *Ptc1* in the chick eye primordium suggest that Shh signaling may play a role during the optic vesicle to optic cup transition. Thus, we have tested this hypothesis by evaluating the temporal requirements for Shh activity *in vivo* at two different stages of chick eye development. The effects of altering Shh signal levels on eye pattern formation and on the expression of genes critical for D-V patterning and for the specification of distinct eye tissues were examined. Our results demonstrate that Shh activity is required for eye morphogenesis during the transition from the optic vesicle to the optic cup, as well as after initial formation of the double-layered optic cup. Our data further reveal that the early vertebrate eye primordium (including the retina, the pigmented epithelium, and the optic stalk) is subdivided into dorsal and ventral compartments that show distinct responses to either the gain or loss of Shh signals. Moreover, altering Shh signal levels caused corresponding changes in *BMP4* expression in the optic cup, suggesting that Shh and BMP4 signals antagonize each other to coordinate D-V patterning of the eye.

MATERIALS AND METHODS

Chick Embryos

White Leghorn chicken eggs were purchased from Spafas, Inc. (Norwich, CT) and incubated at 38°C in a humidified rotating incubator. Developmental stages of embryos were assigned according to 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.* (1993). The parental RCAS(A) virus (Hughes *et al.,* 1987) was used as controls in viral infection experiments. Viral stocks with $1–2 \times 10^8$ CFU/ml titers were prepared by transfecting chick embryonic fibroblast cells with viral DNA constructs, collecting culture media, and concentrating virus by centrifugation as described (Morgan and Fekete, 1996).

Concentrated viral stocks were mixed with a 1/10 vol of 0.25% fast green dye immediately before injection (Morgan and Fekete, 1996). For stage 10 infection, the viral inoculum was injected into the ventricle of the neural tube (10 somites, 33–38 h of incubation) at the junction of the forebrain and midbrain and directly into the optic vesicles until the primary optic vesicles were filled (0.2 to 0.4 µl). For stage 17 injections, the viral inoculum (0.1 to 0.2 µl) was injected into the subretinal space. Eggs were sealed with tape and further incubated 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 (Iscove's modified Dubecco's medium, Gibco BRL) supplemented with 20% fetal calf serum and 2 mM glutamine. The control hybridoma cells producing anti-viral GAG protein IgG antibodies (3C2, Stoker and Bissell, 1987) were cultured in DMEM containing 10% fetal calf serum. For *in ovo* 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. The cells were mixed with a 1/10 vol of 0.25% fast green dye immediately before injection. Hybridoma cells were injected into the ventricle of the anterior neural tube at stage 10 (0.4 μl) and into the vitreal space of the right eye at stage 17–18 (0.2–0.4 μl) using pulled glass pipettes.

Histological Staining

Embryos were fixed in 4% paraformaldehyde overnight and embedded in paraffin following standard procedures; 6-μm sections were dewaxed and stained with hematoxylin and eosin using standard procedures.

RNA Probes

Digoxigenin-labeled RNA probes were generated according to the manufacturer's instruction (Boehringer Mannheim) using chicken cDNA templates. Drs. Randy Johnson (M. D. Anderson Cancer Center, Houston, TX) and Cliff Tabin (Harvard Medical School, Boston, MA) provided Sonic hedgehog (Riddle *et al.,* 1993), Indian hedgehog (Vortkamp *et al.,* 1996), Desert hedgehog, Patched1 (Marigo *et al.,* 1996b), and BMP4 (Oh *et al.,* 1996) cDNAs; Dr. Doris Wu (NIDCD) provided Otx2 cDNA (Wu *et al.,* 1998); Dr. Peter Gruss (Max-Planck-Institute, Gottingen, Germany) provided the chick Pax6 probe (Goulding *et al.,* 1993); and Dr. Constance Cepko provided the chick Vax probe (Schulte *et al.,* 1999).

The 387-bp-long chick Pax2 cDNA clone was generated by RT–PCR using a chick stage 24 cDNA library as templates (Riddle *et al.,* 1993) and degenerate primers XJY25 (5′CGG,GAT,CCA,AY-C,ARY,TIG,GIG,GIG,TIT,TYG,T) and XJY26 (5′GCG,AAT,TCG-,GRT,GRA,AIG,GYT,GYT,GIA,CYT,T). These primers corresponded to the amino acid residues NQLGGVF and KVQQPFHP of the mouse Pax2 protein, respectively. This partial chick Pax2 cDNA is 85% homologous at the DNA level and 97% identical at the protein level to the mouse Pax2 sequences (Dressler *et al.,* 1990).

In Situ Hybridization

Whole mount *in situ* hybridization experiments were performed as described by Riddle *et al.* (1993). More than 6 each of control or viral/hybridoma-treated embryos were coprocessed in the same tube. The hearts of control embryos were removed to facilitate identification. For each cDNA probe, two or more *in situ* hybridization experiments (a total of 12 or more injected embryos) were performed. Gene expression patterns were compared between con-

FIG. 1. Expression patterns of *Shh, Ptc1,* and *BMP4* during chick eye morphogenesis detected by *in situ* hybridization. In all panels, dorsal is up. (A) Partial dorsal view of a stage 11 chick embryo processed by whole mount *in situ* hybridization. High levels of *Shh* expression signal are present between the two optic vesicles. (B) A cross section of stage 10 optic vesicles. *Shh* is expressed in the ventral forebrain, the prechordal mesoderm, and the primitive foregut. (C) A cross section of stage 12 optic vesicles. The most intense Shh hybridization signals appear at the junction of the protruding optic vesicles and the ventral forebrain (arrowheads). (D–E) Adjacent cross sections of a stage 13 head showing (D) *Shh* expression in the ventral midline and lateral walls of the diencephalon and (E) *Ptc1* expression in the ventral and lateral diencephalon as well as the mesenchyme. Note that *Ptc1* expression domains are broader than Shh expression domains. (F–G) Adjacent cross sections of a stage14 head through the optic fissure showing (F) *Shh* expression in the ventral diencephalon, which is continuous with the optic stalk, and (G) *BMP4* expression in the dorsal retina (arrowhead). (H–J) Adjacent cross sections of a stage 18 head showing (H) *Shh* expression in the ventral forebrain, (I) *Ptc1* expression in the diencephalon, the pigmented epithelium (arrowheads), and in the optic stalk and (J) *BMP4* expression in the dorsal retina and the ventral pigmented epithelium (arrowheads). (K–M) Sections of stage 24 eyes showing (K) low levels of *Shh* expression in the inner portion of the central retina (arrowhead), (L) low levels of *Ptc1* expression in the retina (arrowhead) and the optic stalk, and (M) *BMP4* expression in the dorsal peripheral retina and ventral pigmented epithelium (arrowheads). The scale bar in (A) represents 500 μm; scale bars in (B–M) represent 100 μm. pc, prechordal plate; fg, foregut; ov, optic vesicle; di, diencephalon; le, lens placode, or lens vesicle; pe, pigmented epithelium; os, optic stalk; ret, retina.

FIG. 2. Viral-mediated *Shh* expression and anti-Shh antibody production by 5E1 hybridoma cells. (A–F′) Transverse sections of stage 24 eyes infected by viruses at stage 10 are shown. (A) and (B) show sections derived from a Shh virus-infected eye hybridized for *Shh* and *Pct1* antisense probes, respectively. Note that the uneven ectopic expression of *Shh* mRNA (A) leads to a more evenly distributed *Ptc1* mRNA in the adjacent section (B). (C) and (D) show the same cross section derived from a Shh virus-infected eye stained for the anti-viral antibody P27 (C, red) and the anti-Shh antibody 5E1 (D, green), respectively. (E) and (F) show the same section from a control virus RCAS-infected eye stained with P27 (E, red) and 5E1 antibodies (F, green), respectively. (F′) shows an enlarged view of the central retina in (F). Both Shh (C) and control RCAS (E) virus-infected eyes show positive staining for the viral GAG protein; however, Shh virus-infected eyes show Shh protein signals (D) elevated above the endogenous Shh protein staining signals detected in the ganglion cell layer (arrow, F′). (G–L) Sections of stage 27 embryos injected at stage 17 with anti-Shh 5E1 hybridoma cells (H, J, L) or control 3C2 hybridoma cells (G, I, K). All sections were incubated with anti-mouse secondary antibodies (green). 5E1 hybridoma cell-injected embryos show secondary antibody labeling of Shh expressing cells in the zona limitans (H, arrows) and in the ventral midline (J, arrow) of the diencephalon, as well as of 5E1 hybridoma cells in the vitreal space of the eye (L, arrowhead). Production of the control 3C2 antibody is also detected by the secondary antibody (K, arrowhead); however, no labeling of Shh producing cells is detected in the diencephalon (G, I). These data indicate that anti-Shh antibodies are produced by 5E1 cells *in vivo,* and they diffuse and bind to endogenous Shh producing cells. Scale bars in (A–L) represent 100 μm; the scale bar in (F′) represents 25 μm. di, diencephalon; le, lens; pe, pigmented epithelium; os, optic stalk; zl, zona limitans.
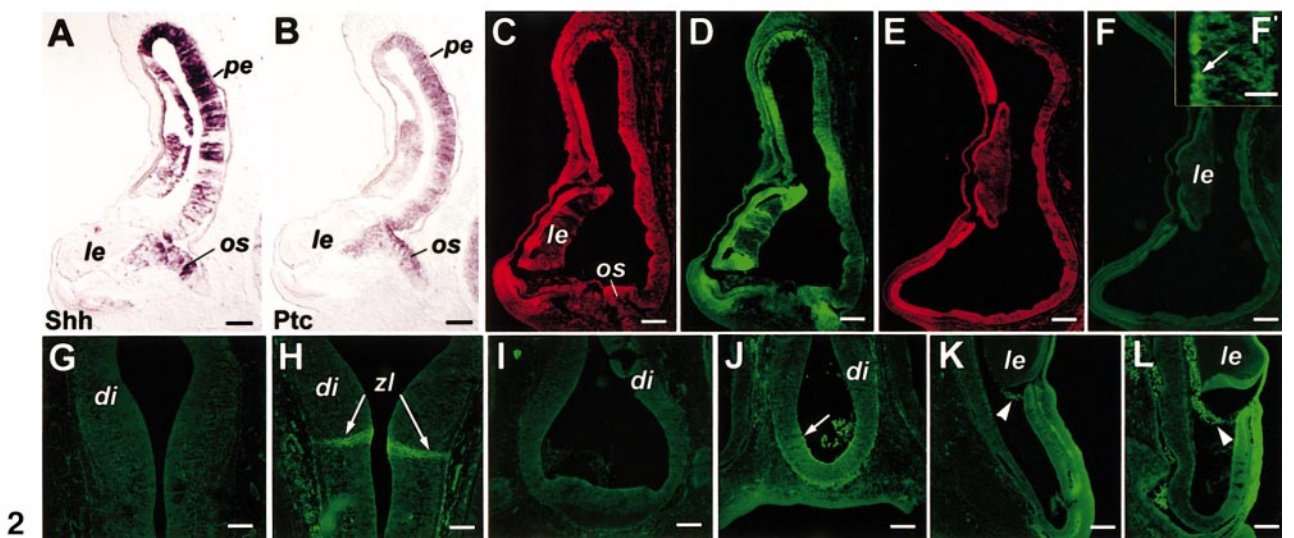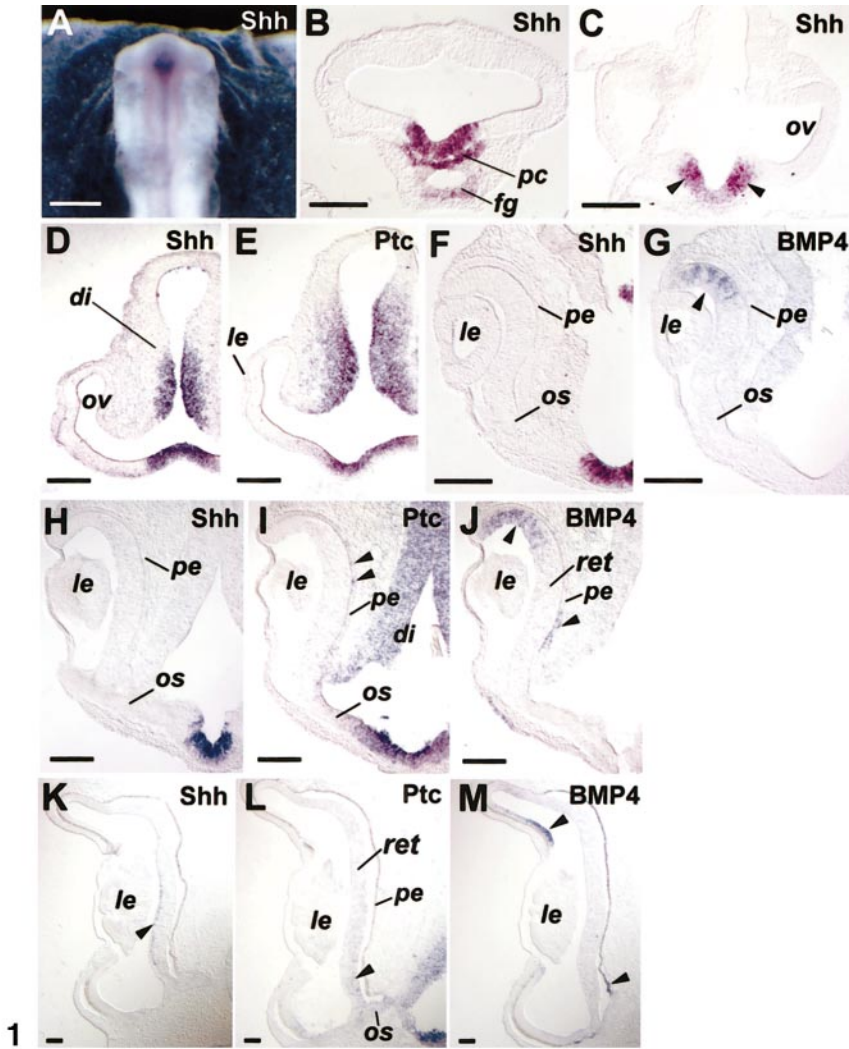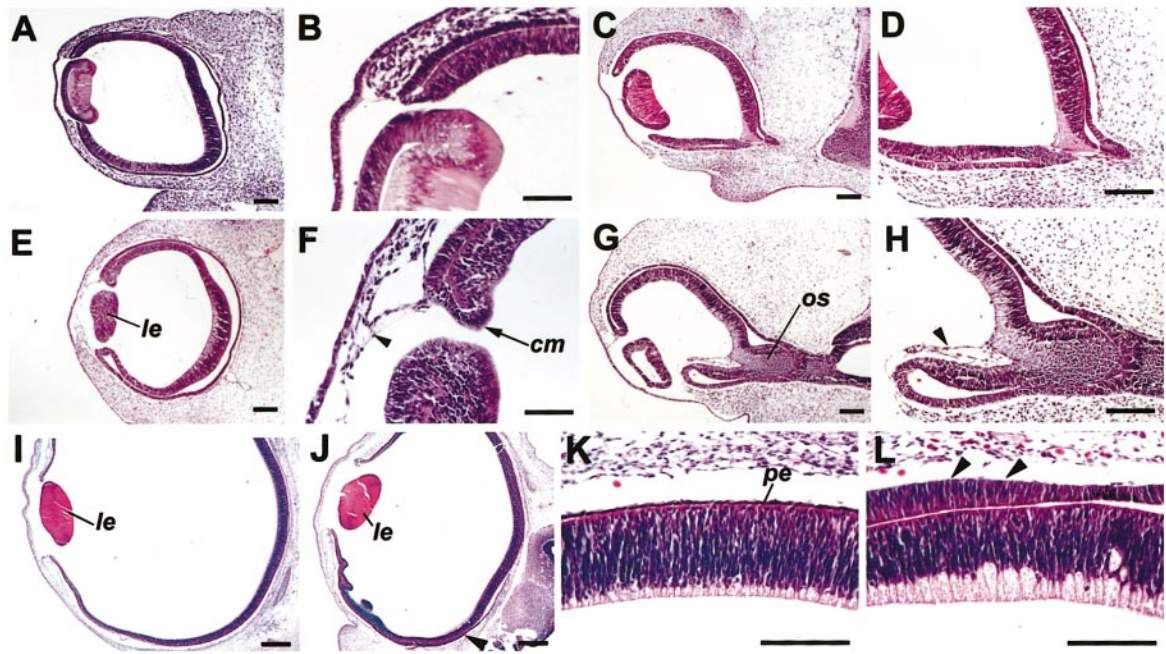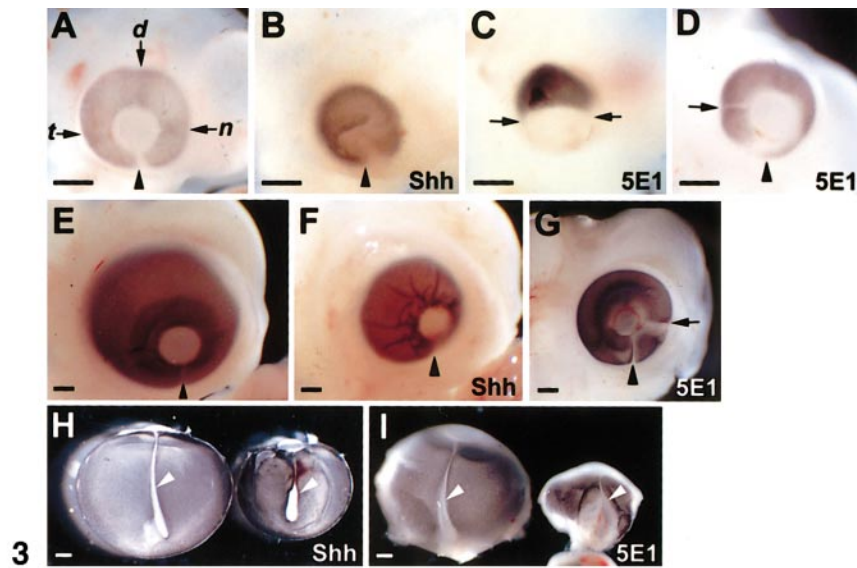
FIG. 3. Effects of perturbing Shh signals on chick eye morphogenesis. External morphology of stage 24 (A–D) and stage 29 (E–I) eyes are shown. (A–G) Lateral views of chick eyes with ventrally positioned embryonic optic fissure (arrowheads). Untreated normal chick eyes at stage 24 (A) exhibit four quadrants demarcated by morphological constrictions (arrows) at the dorsal, nasal, and temporal aspects of the eye and the ventral fissure (arrowhead). These morphological constrictions become less pronounced at stage 29 (E6) in untreated normal eyes (E). Infections with the Shh virus at stage 10 and stage 17 result in small eyes at stage 24 (B) and stage 29 (F), respectively. Injection of anti-Shh antibody producing 5E1 cells at stage 10 leads to the formation of small eyes at stage 24 (C) with the loss of ventral pigmentation (arrows point to the D-V boundary). Injection of 5E1 cells into stage 17 eyes leads to small eyes at stage 24 (D) and stage 29 (G). Note the sharp boundaries associated with the loss of pigmentation (arrows in D, G), revealed by the anti-Shh antibody treatment. Control RCAS virus-infected and control 3C2 hybridoma-injected eyes (not shown) exhibit the same morphology as untreated normal eyes. (H,I) Ventral views of stage 29 eye globes showing the optic fissure region (white arrowheads). (H) Infection by Shh virus at stage 17 leads to a widened optic fissure compared with the control RCAS virus-infected eye (shown on the left). (I) Treatment with 5E1 cells causes an expansion of the nonpigmented area ventrally compared to the control 3C2 antibody-treated eye (shown on the left). Scale bars in all panels represent 500 μm. t, temporal; n, nasal; d, dorsal.

FIG. 4. Effects of altering Shh signals on the histogenesis of ocular tissues. (A–H) Histological staining of stage 23 eyes. Compared to control RCAS virus-infected eyes (A–D), eyes infected by Shh virus at stage 10 (E–H) display optic nerve hyperplasia, ciliary margin defects (F, arrow), malformed lens, and extravitreal cells in the vitreous and in the anterior chamber of the eye (F, H, arrowheads). (I–L) Histological staining of stage 29 eyes injected with hybridoma cells at stage 17. Compared to control antibody 3C2-treated eyes (I, K), anti-Shh antibody 5E1-treated eyes (J, L) are smaller in size, and the ventral pigmented epithelium layer has adopted the appearance of the neural retina (J, L, arrowheads). Scale bars in (A–H, K–L) represent 100 μm; scale bars in (I, J) represent 300 μm. cm, ciliary margin; os, optic stalk; pe, pigmented epithelium; le, lens.

trols (RCAS virus or 3C2 hybridoma) and Shh virus or 5E1 hybridoma-treated embryos that were age-matched based on morphological features of the trunk and limbs.

In situ hybridization experiments using cryosections and digoxigenin-labeled probes were performed according to a modified protocol (Yang and Cepko, 1996) based on the whole mount *in situ* hybridization procedure previously described (Riddle *et al.,* 1993; Wilkinson, 1992), except the hybridization for Shh and Ptc1 at stage 24 was performed at 65°C overnight, followed by two 30-min washes at 65°C in 50% formamide, 1× SSC, and 0.1% Tween 20. For each probe, a minimum of three embryos per experimental condition were sectioned and analyzed.

Immunocytochemistry

For all immunocytochemical staining experiments, a minimum of three eyes per experimental condition of a given stage were analyzed.

Staining with all antibodies except the anti-BrdU antibody (Amersham) was performed using 12- to 14-μm thickness cryosections of tissues fixed with 4% paraformaldehyde. Sections were incubated with primary antibodies and visualized using biotinylated secondary antibodies and the Vectastain ABC Elite kit (Vector Labs) with 3′,3′-diaminobenzidine (DAB) as chromogen or by Texas red-conjugated (Jackson ImmunoResearch Laboratories) or Alexa 488-conjugated (Molecular Probes) secondary antibodies.

Monoclonal antibodies recognizing the Pax6 protein (Ericson *et al.,* 1997) and the islet1 protein (clone 4D5, Yamada *et al.,* 1993) were obtained from DSHB (Iowa City, IA). The polyclonal antibody against Pax2 protein was purchased from BAbCO (Richmond, CA). The anti-viral GAG protein monoclonal antibody 3C2 was produced as described (Stoker and Bissell, 1987). The anti-viral protein polyclonal antibody P27 was purchased from SPAFAS.

For anti-BrdU immunostaining, after denucleation, a 5 mM BrdU stock solution was injected intravitreally to achieve a concentration of ~60 μM in the vitreal space of the eye. The eyes were further cultured in DMEM containing 10% fetal calf serum, 2.5% chick serum, and 60 μM BrdU at 37°C for 3 h in a 5% CO_2 incubator. After incubation, eyes were embedded in OCT (Miles) by quick freezing in liquid N_2. Sections 14 μm in thickness were submerged in 100% methanol at −20°C overnight, air-dried, and then treated with 95% ethanol, 5% acetic acid solution for 2 min, followed by incubation with 2 N HCl for 5 min. After extensive washes using DMEM, the sections were treated with blocking solution containing 0.3% Triton X-100, incubated with the undiluted anti-BrdU antibody containing nuclease (Amersham) at room temperature for 1 h, and detected by the HRP immunocytochemical staining procedure as described above.

Quantification of Cell Numbers

Retinas infected with control RCAS virus ($n = 5$) or Shh virus ($n = 5$) at stage 17 were dissected and dissociated at E6 using the method described by Altshuler and Cepko (1992). Briefly, retinas were incubated with trypsin followed by trituration in the presence of DNase to achieve single-cell suspensions. Cell suspensions were further diluted and directly counted using a cytometer. Total numbers of cells per retina were then calculated. On average control E6 chick retina contains 5×10^6 cells.

TUNEL Assay

Cryosections 14 μm in thickness 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, Ptc1, and BMP4 during Chick Eye Primordium Formation

In order to determine the roles of Shh in chick eye morphogenesis, we examined the expression of *Shh* mRNA during the transition from the optic vesicle to the optic cup. In chick, rudimentary optic vesicles initially become visible at the neural fold stage (HH stage 8.5, 7 somites) (Hamburger and Hamilton, 1951). Whole mount *in situ* hybridization using stage 11 embryos (13 somites) detected an anterior *Shh* expression domain located between the two optic vesicles (Fig. 1A). This anterior *Shh* expression domain was broader than the expression domains of *Shh* in more caudal regions, suggesting that higher levels of Shh signal may be present in this rostral region of the embryo. At stage 10 (10 somites), the expression of *Shh* detected in sections was in a broad area in the ventral forebrain as well as in the prechordal plate and the foregut (Fig. 1B). The optic vesicle at this stage did not show an overt D-V asymmetry at the morphological level; however, by stage 12, a D-V asymmetry was apparent (Fig. 1C). The strongest expression of *Shh* at stage12 was observed in two lateral domains at the base of the protruding optic vesicle, although a relatively weak *Shh* hybridization signal was present in the midline (Fig. 1C). At the beginning of optic vesicle invagination and lens placode thickening, *Shh* mRNA was expressed by the ventral forebrain tissues adjacent to the optic primordium (Fig. 1D, stage 13). Throughout the early optic cup stages, hybridization signals for *Shh* were observed in the forebrain region connected to the ventral optic stalk (Fig. 1F, stage 14; Fig. 1H, stage 18). By stage 24, expression of the *Shh* gene was also detected in the inner portion of the central retina (Fig. 1K), where newly differentiated ganglion cells resided. However, the intensity of hybridization signals suggested that the expression level of *Shh* mRNA in the stage 24 neural retina was significantly lower than its expression level in the ventral forebrain (data not shown). *In situ* hybridizations using probes for Indian hedgehog and Dessert hedgehog did not yield any detectable signals during these early stages of chick eye morphogenesis (data not shown).

Since Shh enhances transcription of its receptor *Patched-1* (*Ptc1*) in cells receiving Shh signals, *Ptc1* expression can be used as an indicator of Shh activity (Marigo and Tabin, 1996a; Goodrich *et al.,* 1996; Goodrich and Scott, 1998). As expected, at the onset of optic vesicle invagination, *Ptc1* mRNA was detected in overlapping yet broader regions of the neural tissue where *Shh* mRNAs were de-

tected, as well as in the head mesenchyme adjacent to the diencephalon (Fig. 1E, stage 13). Upon formation of the optic cup, *Ptc1* hybridization signals were seen in the optic stalk, in the pigmented epithelium, and in the diencephalon (Fig. 1I, stage 18). Furthermore, *Ptc1* mRNA signals showed a medial–lateral spatial gradient that extended from the ventral midline into the optic stalk beyond the region of *Shh* mRNA expression (Fig. 1I), suggesting that Shh protein diffused from its site of synthesis to influence the ventral optic primordium. At stage 24, low levels of *Ptc1* signals were detected in the retinal epithelium and the optic stalk (Fig. 1L), in part reflecting the *de novo* synthesis of Shh molecules by neural retinal cells (Fig. 2F′).

The expression of *BMP4* mRNA was first detected in the chick retina at the onset of the double-layered optic cup formation (stage 13, data not shown). At stage 14, the dorsal retina expressed *BMP4* (Fig. 1G). By stage 18, the dorsal portion of the retinal layer and the ventral portion of the pigmented epithelium layer showed *BMP4* hybridization signals (Fig. 1J). By stage 24, BMP4 expression was detected in the dorsal peripheral retina and ventral pigmented epithelium (Fig. 1M). These expression patterns were consistent with previously reported *BMP4* expression patterns in the mouse eye (Furuta and Hogan, 1998) and chick head (Shigetani *et al.,* 2000).

The expression patterns of *Shh* in the ventral forebrain, as well as the graded expression of its receptor *Ptc1* in the ventral optic primordium, suggest that Shh signals may specify ventral eye characteristics during the transition from the optic vesicle to the optic cup. The restricted dorsal expression of *BMP4* may define dorsal properties of the retina.

Altering Shh Signal Levels during or after Optic Cup Formation Disrupts Eye Morphogenesis and Reveals a D-V Boundary

In order to assess the temporal requirements of Shh activity for eye patterning and ocular tissue specification, we altered endogenous levels of Shh signal by misexpressing the chick Shh protein using a retrovirus (RCAS · Shh, Riddle *et al.,* 1993) and by inhibiting endogenous Shh activity using a Shh-blocking antibody (5E1, Ericson *et al.,* 1996). Perturbations were carried out *in vivo* either when the optic vesicle was formed (HH stage 10) or soon after optic cup formation (HH stage 17). Infection of the developing eye primordium with the Shh virus resulted in ectopic *Shh* mRNA expression in the retina (Fig. 2A). In addition, a corresponding increase in *Ptc1* transcription was observed (Fig. 2B), indicating that Shh signaling levels had increased as a result of Shh virus infection. Furthermore, elevated Shh protein expression was detected in Shh virus (Figs. 2C and 2D) but not control virus-infected eyes (Figs. 2E and 2F). Injection of anti-Shh 5E1 hybridoma cells (Figs. 2H, 2J, and 2L), but not control 3C2 hybridoma cells (Figs. 2G, 2I, and 2K), resulted in labeling of the endogenous Shh producing cells located in the ventral midline and in the

zona limitans of the diencephalon (Figs. 2H and 2J). Thus, anti-Shh antibodies produced by 5E1 hybridoma cells were able to diffuse through the tissues and bind to endogenously expressed Shh proteins.

To determine if maintaining normal levels of Shh signaling was essential for the morphogenic transition from the optic vesicle to the optic cup, Shh virus or anti-Shh 5E1 hybridoma cells were delivered to the anterior neural tube at stage 10, and embryos were analyzed at embryonic day 4 (E4, HH stage 24, 48 h postinjection). The normal chick eye at stage 24 showed signs of morphological constrictions at nasal, temporal, and dorsal positions (Fig. 3A). Together with the ventral optic fissure these constrictions subdivided the developing eye into four quadrants. When *Shh* was misexpressed at stage 10, more than 90% of the infected embryos ($n = 382$) exhibited small, malformed eyes at stage 24 (Fig. 3B). Furthermore, the average distance between the two eyes increased approximately 12%, as measured from the corneal surfaces. Control RCAS virus-infected embryos ($n = 214$) exhibited no morphological defects postinfection. After injection of 5E1 hybridoma cells at stage 10, >95% of embryos ($n = 108$) developed small eyes, which showed a loss of pigmentation in the ventral half of the eye at stage 24 (Fig. 3C). In addition, 5E1 antibody-treated eyes were rotated toward the ventral midline. Thus, both increasing and decreasing Shh signal levels at the time of optic vesicle formation (stage 10) severely affected the morphology of the optic cup.

To determine if Shh signals are required for eye development after formation of the optic cup, Shh virus was injected into the subretinal space and 5E1 hybridoma cells were injected into the vitreal space of the optic cup at stage 17. At embryonic day 6 (E6, stage 29), normal chick eyes showed no obvious morphological constrictions (Fig. 3E). However, greater than 95% of the optic cups infected with Shh virus ($n = 155$) displayed microphthalmia at E6 (Fig. 3F), which was accompanied by an apparent loss of ventral tissue, abnormal foldings of the retina and/or pigmented epithelium, abnormal ciliary margins, and a widened optic fissure (Fig. 3H). Control RCAS virus-infected ($n = 86$) or noninfected eyes on the contralateral side showed no abnormalities. Embryos injected with 5E1 hybridoma cells ($n = 120$) at stage 17 also displayed smaller eyes at stage 24 (Fig. 3D) and stage 29 (Fig. 3G), compared to control hybridoma cell 3C2 (Stoker and Bissell, 1987)-injected eyes ($n = 70$). Interestingly, eyes treated with the Shh absorbing antibody displayed sharp nonpigmented boundaries at stages 24 and 29 (Figs. 3D and 3G) that were not present in control hybridoma-injected or untreated eyes. The positions of these nonpigmented boundaries corresponded to the positions of the morphological constrictions demarcating the four quadrants observed in normal eyes between E4 and E5. Furthermore, loss of pigmentation occurred more frequently in the ventral half of the eye (Figs. 3C, 3D, 3G, and 3I). These results indicate that proper levels of Shh activity are continuously required for eye patterning even after formation of the optic cup. Moreover, the early optic
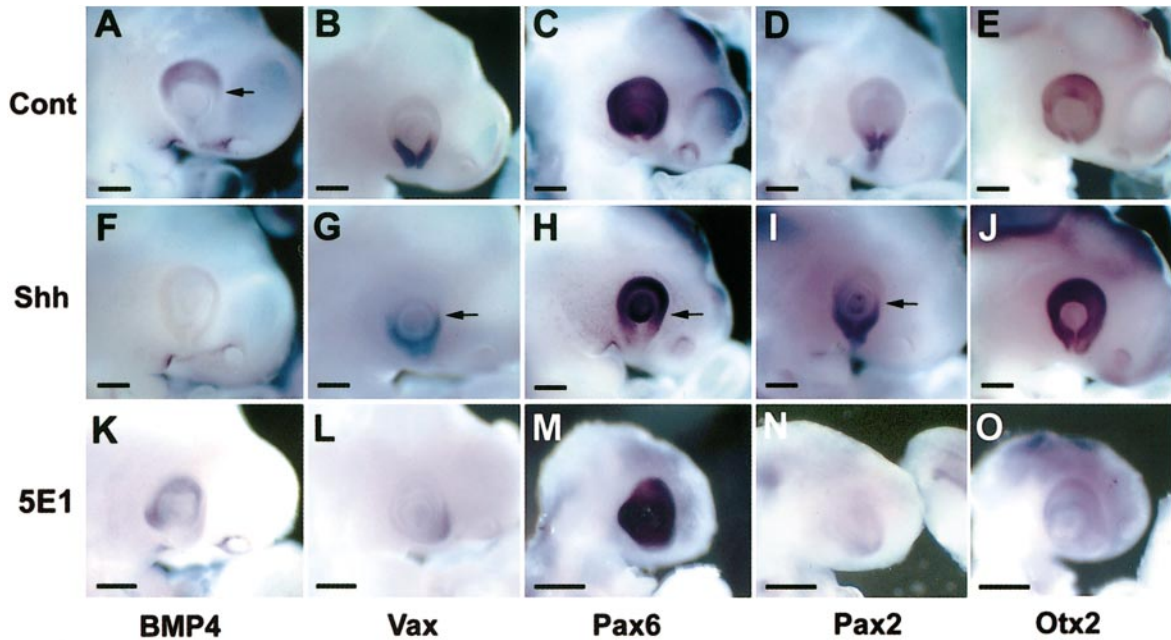
FIG. 5. Altered gene expression patterns caused by perturbation of Shh at the optic vesicle stage. Lateral views of embryos injected at stage 10 with control RCAS virus (A-E), Shh virus (F-J), or 5E1 hybridoma cells (K-O) and assayed by whole mount *in situ* hybridization. All eyes are at stage 22 except those in (M–O), which show stage 19 eyes. Control 3C2 hybridoma cell-injected embryos (data not shown) exhibit patterns of gene expression similar to those of RCAS virus-infected (A–E) or untreated embryos (not shown). Compared to control virus-infected eyes, ectopic *Shh* expression results in dorsal inhibition of *BMP4* (A, F), the limited expansion of *cVax* (B, G) and *Pax2* (D, I), the ventral suppression of *Pax6* (C, H), and the apparent induction of *Otx2* in the eye (E, J). Conversely, decreasing Shh signal levels by 5E1 cell injection causes a ventral expansion of *BMP4* (A, K), a reduction of *cVax* expression domain (B, L), enhanced expression of *Pax6* (C, M), and reduced expression of *Pax2* (D, N) and Otx2 (E, O). The proposed D-V boundaries are indicated by arrows. Scale bars in all panels represent 250 μm.

cup appears to contain a D-V boundary demarcating distinct compartments that show different sensitivities to the reduction of Shh signals.

Histological analyses of eyes infected by Shh virus at the optic vesicle stage (stage 10) revealed that the ventral region of the retina adjacent to the optic stalk had adopted an optic stalk-like appearance (Figs. 4G and 4H). In addition, serial sections showed that the optic stalk was broadened as well as elongated and contained more cells than control RCAS virus-infected eyes. Shh virus-infected eyes also showed abnormal ciliary margins, the presence of extra vitreal cells, and disrupted lens vesicles (Figs. 4F and 4H). Thus, misexpressing Shh at the optic vesicle stage affected the formation of multiple ocular tissues. Histological analyses of anti-Shh antibody-treated optic cups (stage 17) also revealed defective ventral eye patterning. Consistent with the apparent loss of pigmentation, the outer layer of the ventral optic cup assumed a morphology that resembled retinal neural epithelium instead of the single-layered pigmented epithelium (Figs. 4K and 4L). These results demonstrate that endogenous Shh signals are necessary for the proper patterning and tissue specification of the early optic cup.

Differential Gene Regulation in the Developing Eye by Shh

During embryonic development, secreted Shh molecules pattern tissues by specifying cell fates through regulation of gene transcription. We thus examined if elevated or reduced Shh signals affected expression of important morphogens as well as key transcription factors during the transition of the optic vesicle to the optic cup.

The paired domain-containing *Pax6* and *Pax2* genes are normally expressed in distinct regions of the expanding eye primordium; the *Pax6* gene is transcribed in the retina, the pigmented epithelium, and the lens (Fig. 5C), whereas *Pax2* expression is restricted to the optic stalk and the optic fissure at stage 22 (Fig. 5D). Whole mount *in situ* hybridization showed that in Shh virus-infected eyes, the *Pax6* expression domain was reduced in the ventral, but not in the dorsal, eye (Fig. 5H). Conversely, *Pax2* gene expression was expanded dorsally in Shh virus-infected eyes (Fig. 5I). In contrast, 5E1 hybridoma cell-injected embryos showed an enhanced expression of *Pax6* (Fig. 5M) and a loss of the ventral *Pax2* expression domains (Fig. 5N). These dorsal–ventral differential responses of *Pax* gene expression were
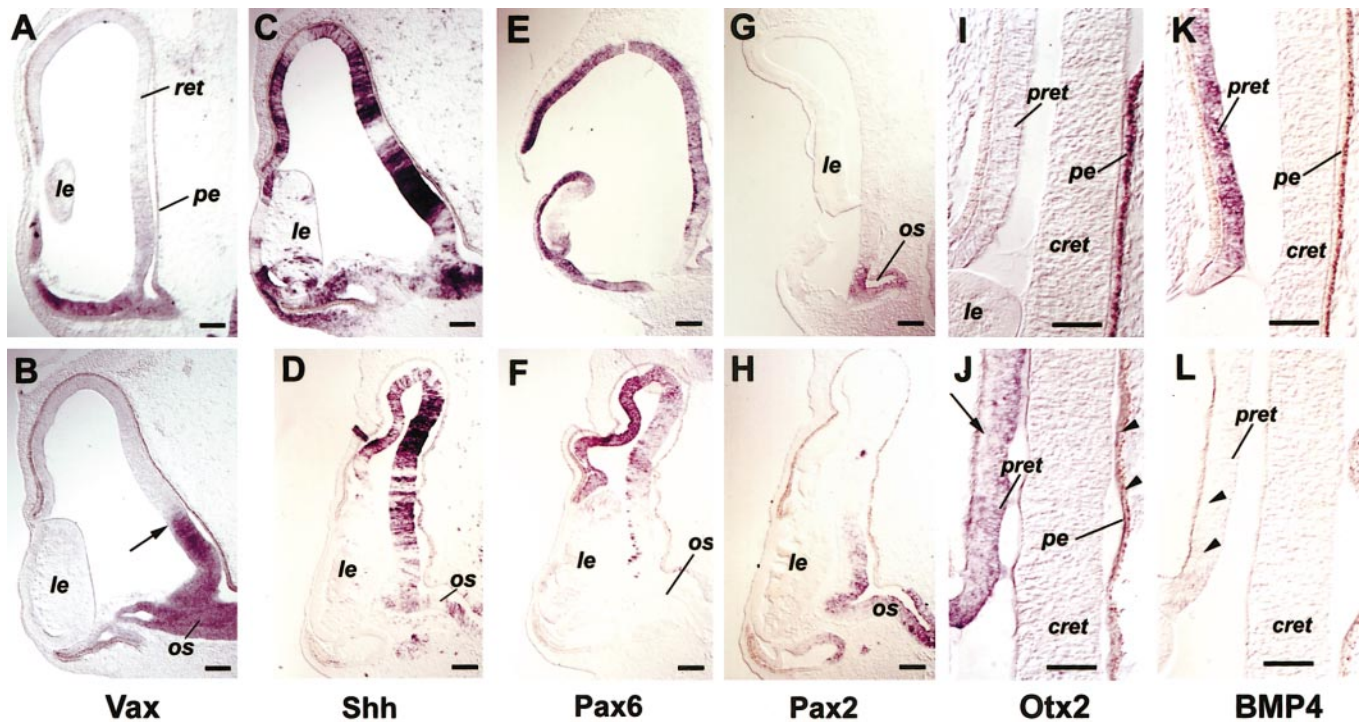
FIG. 6. Differential gene expression patterns in response to ectopic Shh. *In situ* hybridization using coronal sections of stage 24 eyes infected at stage 10 with the control RCAS virus (A, E, G, I, K) and Shh virus (B-D, F, J, L) are shown. Dorsal is up in all panels. (B, C) and (D, F, H) show adjacent sections of the same Shh virus-infected eyes, respectively. (A–C) Expression of *cVax* is enhanced in the ventral retina as a result of ectopic *Shh* expression. Although dorsal retina is infected by the Shh virus (C), *cVax* expression is not induced in the dorsal retina (B). Instead, cVax expression is enhanced in the ventral retina compared to the control (A) and remains restricted to (arrow in B points to the observed D/V boundary) the ventral retina and optic stalk. (E, F) Expression of *Pax6* is reduced in the ventral retina and the lens under the influence of ectopic Shh. Note that Pax6 expression remains in the dorsal retina (F), which is infected by the Shh virus (D). (G, H) *Pax2* expression is expanded from the optic fissure and the optic stalk dorsally under the influence of ectopic Shh. Note that *Pax2* is not induced in the dorsal portion of the retina (H) despite the viral-mediated misexpression of *Shh* in this region (D). (I, J) *Otx2* expression is primarily detected in the pigmented epithelium at stage 24 (I). Ectopic Shh suppresses *Otx2* expression in the central pigmented epithelium (J, arrowheads) and induces *Otx2* in the peripheral retina (J, arrow). (K, L) *BMP4* expression is normally detected in the dorsal peripheral retina at stage 24 (K). Viral-mediated *Shh* expression abolishes this dorsal *BMP4* expression (L, arrowheads). Scale bars in (A–H) represent 100 μm; scale bars in (I–L) represent 50 μm. le, lens; os, optic stalk; ret, retina; cret, central retina; pret, peripheral retina; pe, pigmented epithelium.

not due to limited infection by the Shh virus. *In situ* hybridization using adjacent sections of the same eye demonstrated that despite heavy infection by the Shh virus in the dorsal retina (Fig. 6D), *Pax6* expression was predominantly suppressed in the ventral half of the retina, whereas dorsal expression of *Pax6* was not significantly affected (Figs. 6E and 6F). In adjacent sections of the same eye, *Pax2* expression was enhanced in the ventral retina where *Pax6* transcription was reduced (Fig. 6H), however, Shh did not induce ectopic *Pax2* expression in the heavily infected dorsal retina (Figs. 6G and 6H). These results show that Shh differentially regulates Pax gene expression in the ventral but not dorsal retina.

Since the homeoboxing gene *cVax* acts as an important determinant of the ventral properties of the retina, we next examined how *cVax* gene expression was regulated by Shh signals. In control RCAS virus-infected eyes, whole mount

in situ hybridization showed *cVax* expression in the ventral eye at stage 22 (Fig. 5B), while section *in situ* hybridization revealed high levels of *cVax* mRNA in the ventral-peripheral retina and lower levels of cVax mRNA in the ventral-central retina at stage 24 (Fig. 6A) (Schulte *et al.,* 1999). Viral-mediated Shh overexpression enhanced expression of *cVax* in the ventral eye (Fig. 5G). In contrast, reducing endogenous Shh signals diminished *cVax* expression in the ventral eye (Fig. 5L). Hybridization of adjacent sections derived from the same eye further indicated that although the dorsal retina was infected with the Shh virus (Fig. 6C), the enhancement of *cVax* mRNA transcription was restricted to the ventral half of the retina (Fig. 6B). This result clearly demonstrates that elevated Shh signals positively regulate *cVax* expression in the ventral retina but not in the dorsal retina, again revealing distinct D-V responses of the retina to Shh.

We next examined if altering Shh levels affected expression of the homeodomain-containing *Otx2* gene implicated in eye development (Matsuo *et al.,* 1995; Ang *et al.,* 1996; Acampora *et al.,* 1995; Bovolenta *et al.,* 1997). During normal development of the optic cup, *Otx2* expression is dynamic and is restricted to the pigmented epithelium at around stage 24 (Fig. 6I) (Kablar *et al.,* 1996; Bovolenta *et al.,* 1997). Whole mount *in situ* hybridization suggested an overall increase in *Otx2* expression in Shh virus-infected eyes (Figs. 5E and 5J) and a decrease of Otx2 in 5E1 antibody-treated eyes (Figs. 5E and 5O). Further analyses using sections revealed that the expression of *Otx2* in the pigmented epithelium, especially in the central region, was significantly reduced by *Shh* misexpression, whereas *Otx2* was ectopically induced in the peripheral retina (Fig. 6J), which may have a dominant impact on cell fate (see below).

Together, these results show that perturbing Shh signal levels affect several transcription factors critical for eye patterning and ocular tissue specification. Furthermore, the differential D-V regulation of *Pax6, Pax2,* and *cVax* by Shh supports the existence of dorsal and ventral compartments in the early optic cup.

Antagonizing Effect of Shh Signals to BMP4 Expression

Although dorsal expression of the *Pax* genes was not altered significantly by high levels of Shh, the enhanced transcription of *Ptc1* in the Shh virus-infected retina (Fig. 2B) suggested that the dorsal retina indeed received and responded to ectopic Shh signals. We therefore examined if the dorsally expressed morphogen BMP4 was affected by the level of Shh signaling in the developing optic primordium.

At the onset of optic cup formation, *BMP4* expression was detected in the dorsal retina (Fig. 1G, stage 14), but by stage 22, its expression was more limited to the dorsal peripheral retina (Figs. 5A and 6K). Infection of optic vesicle at stage 10 with the Shh virus completely abolished the dorsal expression of *BMP4* (Figs. 5F and 6L). Thus, the dorsal retina can indeed respond to high levels of ectopic Shh signal. Conversely, injection of 5E1 cells into the optic vesicle at stage 10 caused a ventral expansion of *BMP4* expression domain in the eye (Fig. 5K). Since BMP4 has been shown to positively control dorsal properties of the retina (Koshiba-Takeuchi *et al.,* 2000), Shh-dependent changes in the *BMP4* expression domain may affect the D-V patterning of the retina. Consistent with this, expression patterns of the ventral determinant gene *cVax* correlated with Shh-induced changes in *BMP* expression (Figs. 5G and 5L). These results suggest that elevated Shh signals suppress *BMP* expression, and endogenous Shh signals are necessary to restrict the BMP expression territory. Shh may thus negatively influence the spatial distribution of BMP4 activity in the developing optic primordium.

Altering Levels of Shh Signal Affected Ventral Eye Morphogenesis and Tissue Specification

Since either the gain or loss of Shh signals resulted in severe malformation of the ventral eye, we further characterized the relationship between altered gene expression patterns and the observed tissue defects.

Misexpressing Shh by viral infection at stage 17 resulted in a great reduction of the ventral retina and pigmented epithelium, which lead to a close proximity of the optic nerve head and the ventral ciliary margin (Figs. 7E and 7F). In contrast, the dorsal retina and pigmented epithelium were only slightly reduced. This differential morphogenetic defect was not due to preferential infection of the Shh virus to the ventral eye, since anti-viral 3C2 antibody staining showed that the dorsal and ventral portions of the retina were similarly infected (Fig. 7B). In addition, the optic nerve head of the Shh virus-infected eye was enlarged and the optic nerve deformed (Figs. 7G and 7H). Consistent with this phenotype, the normally restricted distribution of Pax2 protein in the optic nerve head and the optic nerve (Figs. 7E and 7G) was expanded to broad ventral regions (Figs. 7F and 7H). Complementary to the ventral expansion of the Pax2 expressing domain, Pax6 protein expression in the ventral eye was reduced (Figs. 7I and 7J). However, the expression pattern of Pax6 protein in the dorsal retina was largely maintained (Figs. 7I and 7J). Analyses using serial sections confirmed that altered Pax2/6 protein expression was limited to a narrow region (about 500 μm wide) near the optic fissure and surrounding the optic nerve head at E6. These data suggest that the phenotypic small eye at E6 was likely due to the selective disruption of ventral *Pax* gene expression by ectopic Shh, which consequently caused a loss or failed expansion of ventral ocular tissues and/or a retinal to optic stalk cell fate switch by E6.

In contrast to Shh overexpression, inhibiting Shh activity at stage 17 resulted in an exclusion of Pax2 protein from the optic nerve head and the optic nerve at E6 (Figs. 8A and 8E). Residual Pax2-positive cells were displaced to ectopic positions surrounding the optic nerve (Fig. 8E). Conversely, Pax6 protein expression was detected in the optic nerve (Figs. 8B and 8F), which was normally devoid of Pax6 (Fig. 7K). Furthermore, immunostaining with the anti-islet1 antibody (4D5, Yamada *et al.,* 1993), a ganglion cell marker during early retinal neurogenesis (Fig. 8I), detected islet1-positive cells in the prospective optic stalk region of 5E1 antibody-treated eyes (Figs. 8J and 8K), indicating that the optic stalk tissue had adopted a retinal fate.

In addition to ventral optic cup reduction, Shh misexpression also resulted in the appearance of pigmented cells in the retinal layer of the ciliary margin (Figs. 7C and 7D), indicating a cell fate switch from the retina to the pigmented epithelium. This cell specification defect correlated with the ectopic induction of *Otx2* in the peripheral retina by Shh (Fig. 6J). Under decreased Shh conditions, expression of the *Otx2* gene in the ventral outer layer of the optic cup was reduced at stage 24 (Figs. 8G and 8H). Corresponding to

the loss of *Otx2* expression, cells in the outer layer of the optic cup lost pigmentation (Figs. 8G and 8H) and expressed Pax6 protein (Fig. 8F). Moreover, the nonpigmented outer layer of the optic cup stained positive for the Islet1 marker (Fig. 8L), thus confirming the ventral cell fate switch from the pigmented epithelium to the neural retina. Judging by the location of the islet1-positive cells, this duplicated retinal layer displayed an opposite polarity to the normal retina derived from the inner layer of the optic cup. These data thus support a role of *Otx2* in pigmented epithelium specification.

Together, these results indicate that after formation of the optic cup, proper patterning and growth of the ventral optic cup remain sensitive to the levels of Shh signal, which may directly or indirectly regulate transcription factors critical in ocular tissue specification.

Effects of Shh Activity on Retinal Cell Proliferation and Cell Death

Altered levels of Shh at either the optic vesicle or the optic cup stages resulted in microphthalmia in chick, suggesting that Shh signals may have affected cell proliferation and/or cell death. Since Shh virus-infected retinas contained folded areas, we determined if Shh virus-infected retinas had fewer cells by dissociating and counting retinal cells. Results of quantification indicated that retinas infected by Shh virus at stage 17 ($n = 5$) contained on average 40% fewer cells than the control RCAS virus-infected retinas ($n = 5$) at E6.

Effects of Shh on cell proliferation were further analyzed by anti-BrdU immunocytochemical staining. Serial sections of E6 eyes infected at stage 17 showed no significant changes of BrdU labeling patterns in the retina, except for a marked decrease of BrdU-positive cells in a narrow ventral region adjacent to the optic fissure (Figs. 9C and 9D). This region corresponded to the area where altered *Pax* gene expression was observed at E6. Thus, the decreased eye size may be partly due to a reduction in ventral cell proliferation between stage 17 and stage 29 (E6). Similar BrdU labeling assays of eyes injected with 5E1 cells revealed a significant increase of BrdU-positive cells in the outer layer of the ventral optic cup, where the pigmented epithelium was converted to neural retina (Figs. 9E and 9F). However, no significant changes in BrdU labeling patterns were detected in the inner retinal layer compared to controls that were 3C2 hybridoma cell-treated despite the overall reduction of eye size due to the influence of the anti-Shh antibodies.

The effect of Shh dosage on retinal cell apoptosis was examined by TUNEL assays (Gavrieli *et al.,* 1992). In control RCAS virus-infected (data not shown) and in noninfected eyes, only a few apoptotic cells were located in the ventral region of the retina near the optic stalk at stage 24 (Fig. 10A). In contrast, a large number of apoptotic cells were detected in eyes infected by the Shh virus at the optic vesicle stage (Fig. 10B). Moreover, the majority of apoptotic cells were distributed in the ventral retina around the optic

nerve head. Anti-Shh 5E1 antibody-treated eyes also showed an increase in apoptotic cells concentrated in the ventral retina (Fig. 10D), whereas control hybridoma cell (3C2)-injected eyes showed distributions of apoptotic cells near the optic nerve (Fig. 10C) similar to those of noninjected eyes. These results suggest that the reduced eye size caused by Shh overexpression or by Shh-absorbing antibodies may be due in part to an increased level of apoptosis, especially in the ventral retina.

DISCUSSION

Sources of Shh Signal and Its Function in Pattern Formation of the Optic Cup

The transition from the optic vesicle to the optic cup is a critical period of eye patterning since distinct ocular tissues are specified and the D-V asymmetry of the eye becomes further established. The results of our expression studies in chick demonstrate that the main source of Shh signal in the anterior embryo during this morphogenetic process is the ventral forebrain. This conclusion is also supported by the observation that the ablation of diencephalic cells expressing *Shh* similarly affects D-V patterning of the eye (Huh *et al.,* 1999). At the onset of asymmetric D-V growth (stage 12), *Shh* expression at the bases of the protruding chick optic vesicles coincides with the initial appearance of *Pax2* expression in the ventral optic primordium (Schutle *et al.,* 1999; Xiang-Mei Zhang and Xian-Jie Yang, unpublished). Furthermore, dorsally restricted *BMP4* expression emerges at stage 13 in the optic primordium. These spatial and temporal expression patterns are consistent with a model in which ventral midline-derived Shh and dorsal optic cup-derived BMP4 signals play roles in establishing the D-V polarity of the optic primordium.

The ventral forebrain-derived Shh signal may directly or indirectly control optic primordium patterning. During the transition from the optic vesicle to the optic cup, Shh mRNA is expressed by the ventral forebrain tissues, but is not detected in the developing optic stalk. However, the expression pattern of *Ptc1* suggests that Shh signals act at a distance from its site of synthesis to influence ventral eye patterning. From stage 13, at the onset of the optic vesicle invagination, to stage 18, when the double-layered optic cup is formed, *Ptc1* mRNA is detected as a medial to lateral gradient in the ventral forebrain, the surrounding mesenchyme, and in the optic stalk. Thus the proximal region of the optic primordium, the optic stalk, is continuously exposed to Shh signaling. At present, the exact distance of Shh protein diffusion is not known, partly due to the difficulty of detecting low levels of this signaling protein (reviewed by Goodrich and Scott, 1998). Since we do not detect *Shh* or *Ptc1* transcripts by *in situ* hybridization in the optic cup proper before the onset of retinal neurogenesis (stage 20), Shh signals may either act at long range and low concentration to influence ventral optic cup patterning or
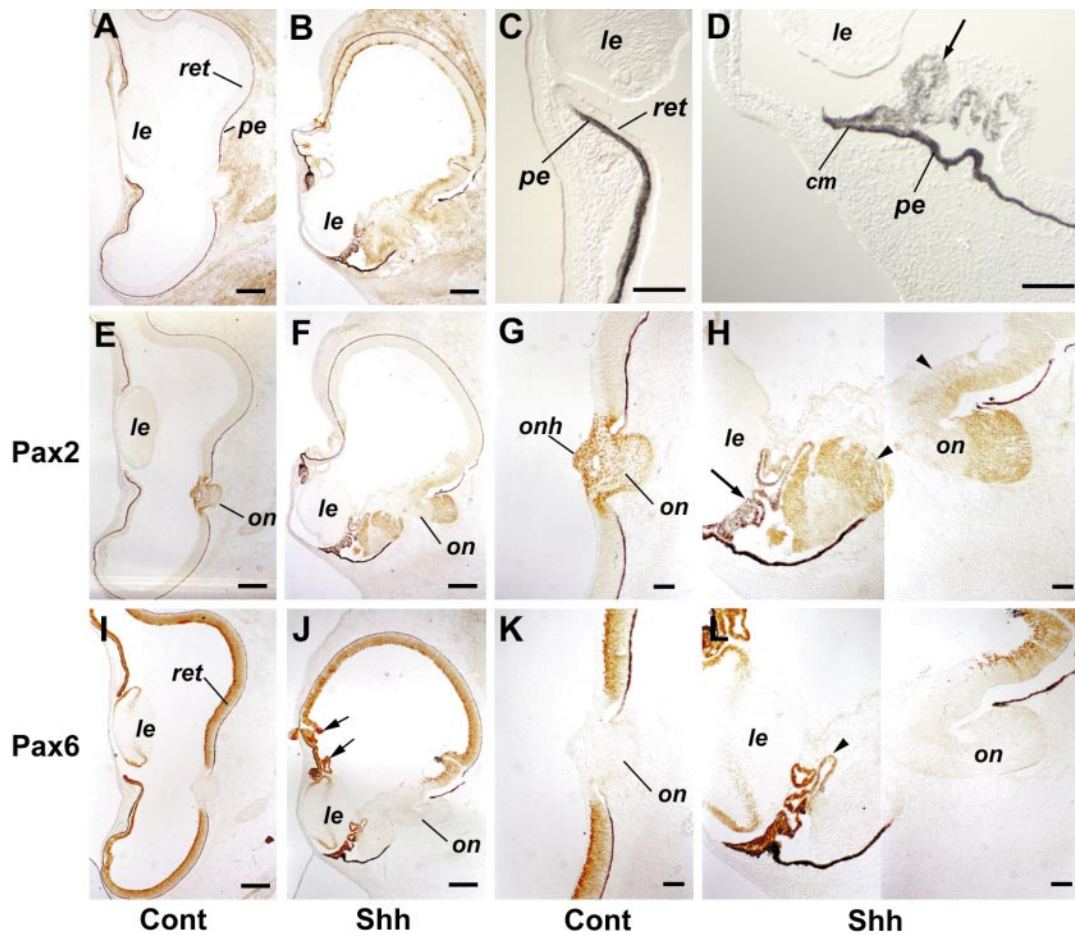
FIG. 7. Effects of Shh overexpression on eye morphogenesis. Immunocytochemical staining of stage 29 (E6) eye sections are shown. (A, C, E, G, I, K) are sections of the noninjected left (contralateral) eye, and (B, D, F, H, J, L) are sections of Shh virus-infected right eye from the same embryo injected at stage 17. (G, H, K, L) are high-magnification views from the optic nerve head regions of (E, F, I, J), respectively. (C) and (D) show noninfected and Shh virus-infected peripheral retinas of nonstained cryosections, respectively. (A, B) Anti-viral 3C2 antibody staining shows that the entire retina of the injected right eye was infected by the Shh virus (B), whereas no viral infection was detected in the noninfected contralateral eye (A) of the same embryo. (C, D) Pigmented cells are present in the peripheral retina of Shh virus-infected optic cup (D, arrow) and are absent in the ciliary margin of the noninfected contralateral eye (C), suggesting a retina to pigmented epithelium fate switch. (E–H) Pax2 antibody staining shows that the Pax2 expression domain has expanded in Shh virus-infected eye. In the noninfected control eye (E, G), Pax2 is limited to the optic nerve head and optic nerve. In the Shh virus-infected eye (F, H), Pax2 protein is detected in broad ventral regions (H, arrowheads). The optic nerve head is clearly enlarged (G, H). (I–L) Pax6 antibody staining indicates that Pax6 protein is greatly reduced in the residual ventral eye tissues infected by Shh virus. In the noninfected control retina (I), Pax6 is devoid of the optic nerve head, but is expressed in the entire retina. The Shh virus-infected eye (J, L) shows reduced Pax6 protein staining in the ventral retina. The ventral ciliary margin portion of the retinal layer, where pigmentation is found (D, H, arrows), remains positive for Pax6 (arrowhead, L). Folding of retina is also found in Shh virus-infected eyes (arrows, J). Scale bars in (A, B, E, F, I, J) represent 300 μm; scale bars in (C, D, G, H, K, L) represent 100 μm. cm, ciliary margin; ret, retina; pe, pigmented epithelium; on, optic nerve; onh, optic nerve head; le, lens.

impact the ventral optic cup indirectly through secondary signals produced by the proximal optic stalk tissue.

Accumulating evidence suggests that the *Hedgehog* family of molecules play multiple roles during vertebrate eye morphogenesis and neurogenesis (Jensen and Wallace, 1997; Levine *et al.,* 1997; Wallace and Raff, 1999; Stenkamp *et al.,* 2000; Neumann and Nuesslein-Volhard, 2000). For ex-

ample, multiple hedgehog genes are expressed by retinal pigmented epithelium and play roles in photoreceptor cell differentiation (Levine *et al.,* 1997; Stenkamp *et al.,* 2000). Interestingly, we also observed *Ptc1* expression in the pigmented epithelium in the early chick optic cup (stage 18). Since we did not detect *Shh, Indian hedgehog,* and *Desert hedgehog* expression in ocular tissues by *in situ*
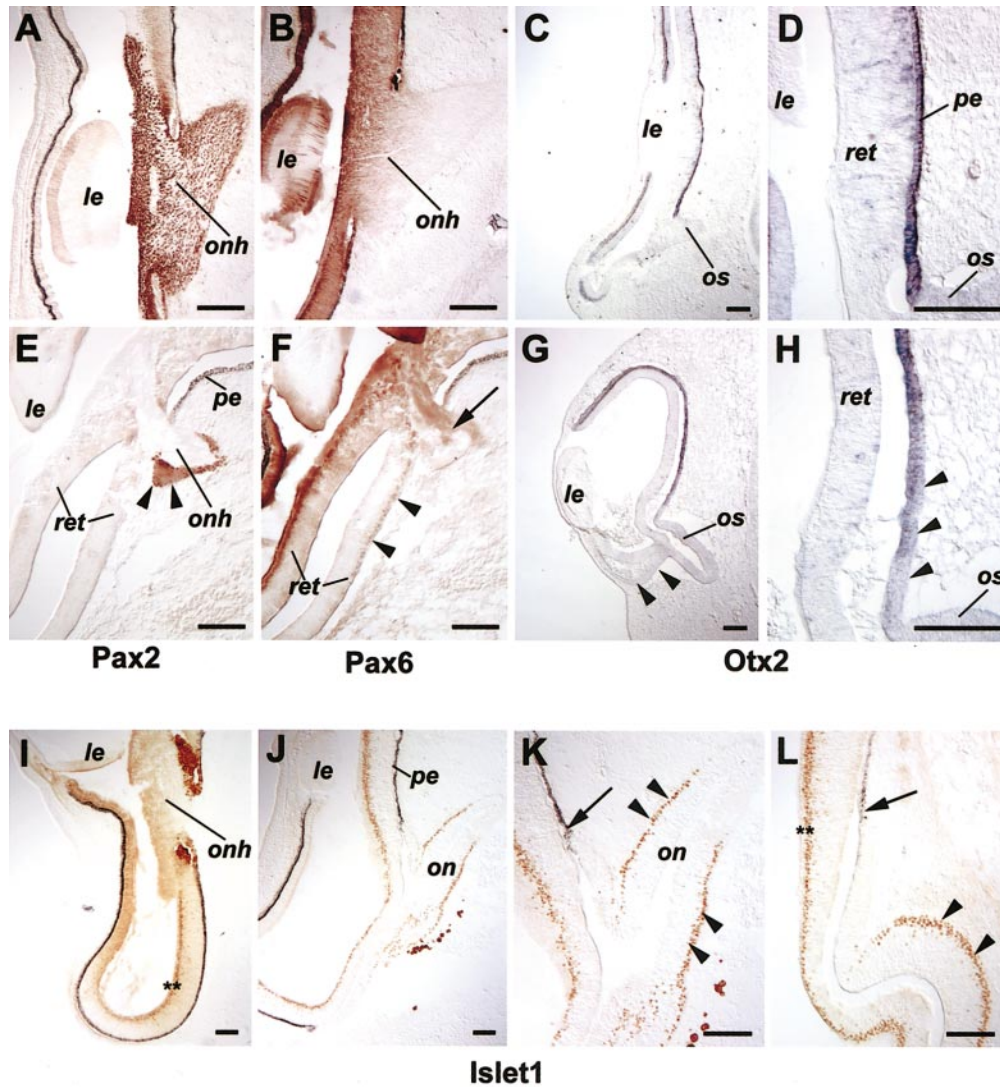
FIG. 8. Effects of reducing Shh signal levels on tissue specification. Sections of eyes injected at stage 17 with control 3C2 (A–D, I,) and 5E1 (E–H, J-L) hybridoma cells are shown. (A, E) Pax2 antibody staining of stage 29 sections shows that Pax2-positive cells are excluded from the optic nerve head under the influence of anti-Shh 5E1 antibodies (E, arrowheads). (B, F) Pax6 antibody staining of stage 29 sections shows that Pax6 protein expression is expanded to the optic nerve (F, arrow) and to the outer layer of the ventral optic cup in anti-Shh 5E1 antibody-treated eyes (F, arrowheads). (C, D, G, H) *Otx2 in situ* hybridization shows that *Otx2* expression in the outer layer of the ventral optic cup is lost by stage 24 under the influence of anti-Shh antibodies (G, arrowheads). The decreased *Otx2* expression correlates with the loss of pigmentation in the pigmented epithelium layer (H, arrowheads). (I–L) Immunocytochemical staining of stage 29 sections with the anti-islet1 antibody 4D5 positively marks the differentiated retinal ganglion cells (indicated by ** in I, L). Injection of 5E1 hybridoma cells induces ectopic islet1-positive cells in the optic nerve (arrowheads in K) and in the outer layer of the ventral optic cup (L, arrowheads), indicating the switch of these tissues to a neural retinal fate. The transition points from the pigmented epithelium to the retina are located dorsal to the optic nerve head (arrows in K, L). Scale bars in all panels represent 100 μm. ret, retina; pe, pigmented epithelium; os, optic stalk; on, optic nerve; onh, optic nerve head; le, lens.

hybridization at this early stage, the induction of *Ptc1* in the pigmented epithelium layer of the optic cup may be due to low levels of Shh diffused from the ventral forebrain. Consistent with previous reports in mouse and zebrafish (Jensen and Wallace, 1997; Neumann and Nuesslein-Volhard, 2000), we also find that after the onset of retinal neurogenesis (stage 24), newborn ganglion cells produce a low level of Shh, which causes a corresponding increase of *Ptc1* mRNA in the retina (Zhang and Yang, 2001). These data suggest that expression of hedgehog genes during

vertebrate eye development is complex and dynamic, and graded Shh signaling is extremely crucial for tissue patterning and cell fate determination.

Shh Signals Are Required throughout Morphogenesis of the Early Optic Cup

Previous studies using targeted Shh gene disruption in mouse (Chiang *et al.,* 1996) or Shh overexpression in one to four cell zebrafish embryos (Macdonald *et al.,* 1995; Ekker *et al.,* 1995) have revealed important functions of Shh in separating the bilateral vertebrate eye fields and in patterning the proximal-distal (D-V) axial aspects of the eye. In this study, we further examined the temporal requirements for Shh signals during chick eye morphogenesis by perturbing anterior Shh signals at two later morphogenetic stages. In viral-mediated misexpression experiments, integration of the viral genome and expression of viral-encoded genes occurs approximately 10–12 h after infection, whereas production of anti-Shh antibodies presumably occurs immediately after hybridoma cell injection. Thus, both types of perturbation at the optic vesicle stage (stage 10, 36 h of incubation) are likely to result in altered Shh levels before or at the time of optic vesicle invagination (stage 13, 48 h of incubation). Our results show that infection with Shh virus and injection with 5E1 cells at stage 10 result in malformed eyes, suggesting that proper levels of Shh signaling are necessary during optic cup formation beyond the stage of initial segregation of the bilateral eye fields. Our results also demonstrate that altering levels of Shh activity at stage 17, when the presumptive retina, pigmented epithelium, and optic stalk became morphologically distinct, caused severe morphological defects in the ventral eye. Therefore, proper Shh signaling is continuously required for pattern formation of the early optic cup, especially for the differentiation of ventral ocular tissues. Moreover, these results show that normal identities of ventral (proximal) ocular tissues, including the optic stalk, pigmented epithelium, and neural retina, can be influenced by environmental signals even after their initial fate specification.

The Existence of Distinct Dorsal and Ventral Eye Compartments

Increasing numbers of genes are found to be expressed differentially along the D-V axis of the vertebrate optic primordium (Bao and Cepko, 1997; Schulte *et al.,* 1999; Koshiba-Takeuchi *et al.,* 2000; Furuta and Hogan, 1998; Fuhrmann *et al.,* 2000). However, the existence of distinct dorsal–ventral compartments in the developing vertebrate eye has not been established. Several lines of evidence from our study suggest that such dorsal and ventral divisions may exist during early stages of optic cup morphogenesis. First, reducing endogenous Shh activity by Shh-blocking antibodies consistently reveals an abrupt loss of pigmentation at a fixed level along the D-V axis of the optic cup, suggesting the existence of a D-V boundary sensitive to Shh

signal levels. The positions of these boundaries correspond to morphological constrictions observed in the normal developing optic cup between stages 22 and 25. Second, ectopic Shh expression alters *Pax 6, Pax2,* and *cVax* transcription in the ventral eye, but not in the dorsal eye. In addition, these changes in gene expression patterns occur in similar domains of the ventral eye. Thus, the dorsal and ventral portions of the early optic cup are distinct at the molecular level as they demonstrate distinct responses to changing levels of Shh. Third, altering Shh signal levels leads to patterning and tissue specification defects in the ventral eye. These cell fate changes occur at a similar D-V axial level relative to Shh-induced changes in gene expression and the D-V boundary induced by reducing endogenous Shh signals.

Based on these data, we speculate that the vertebrate optic primordium at the early optic cup stage consists of a single morphogenetic field that extends from the optic stalk to the dorsal rim of the double-layered optic cup. This morphogenetic field may be subdivided into distinct dorsal and ventral compartments by a D-V boundary, which corresponds to the position of the morphological constriction observed in chick eyes between stages 22 and 25 and demarcates regions with different sensitivities to Shh signals. This D-V boundary is likely to be established soon after optic vesicle invagination (stage 13–14) in both layers of the optic cup, since perturbing Shh signals at the optic vesicle stage (stage 10) resulted in differential D-V gene expression in the retina layer and cell fate switch in the pigmented epithelium layer. Further molecular and cellular analyses are necessary to confirm the existence of these postulated dorsal and ventral compartments in vertebrate eye primordium and to determine the mechanism by which this D-V boundary is established.

Shh Signals and Ocular Tissue Specification

Perturbing Shh signals during the optic vesicle to optic cup transition and after optic cup formation leads to changes in the expression patterns of several transcription factors involved in ocular tissue specification. Modulating Shh activity has opposite effects on expression patterns of *Pax2* and *Pax6* in the optic primordium. These results are in part consistent with a previously described model in which high concentrations of Shh induce *Pax2* expression and define the proximal eye domain consisting of the optic stalk, while low concentrations of Shh permit expression of *Pax6* and specify the distal eye domain including the retina and pigmented epithelium (Macdonald *et al.,* 1995; Ekker *et al.,* 1995). However, despite the spatially unrestricted perturbation that was carried out in chick embryos, the altered gene expression and morphological disruptions that we have observed are mostly limited to the ventral optic primordium. This spatial differential response to Shh signals is likely due to the time at which the perturbations were performed in chick, which were relatively late compared to the overexpression performed in zebrafish. Our

results suggest that before the effective perturbation has occurred in chick (stage 13), the dorsal eye compartment has established specific mechanisms which render the dorsal optic cup insensitive to Shh-dependent regulation of the *Pax* and *Vax* genes.

Overexpression of Shh causes formation of small eye with severe loss of ventral tissues associated with reduced cell proliferation and increased cell death. These phenotypes can be explained by the altered ventral expression of *Pax6* and *Pax2. Pax6* mutations are known to cause growth arrest, failed proliferation, improper differentiation, cell death, and consequently small eye (microphthalmia) or no eye (anophthalmia) phenotypes in mice and humans (Hogan *et al.,* 1988; Hill *et al.,* 1991; Glaser *et al.,* 1994; Quinn *et al.,* 1996). Mutations in the *Pax2* gene result in optic nerve hypoplasia and failure of the optic fissure closure (coloboma) in mouse and human, indicating that *Pax2* plays an important role for the optic stalk determination and ventral fissure development (Sanyanusin *et al.,* 1995; Torres *et al.,* 1996; Schwartz *et al.,* 2000). Thus, loss of *Pax6* expression and expansion of Pax2 expression in the ventral optic cup caused by ectopic Shh may result in a partial or complete switch of the ventral retinal fate to the optic stalk fate, which may consequently lead to increased cell death and abnormal cell proliferation. Conversely, blocking endogenous Shh activity results in the loss of *Pax2* expression in the perspective optic stalk tissues and the complementary expansion of the *Pax6* expression domain, which may be sufficient to alter the fate of optic stalk cells to retinal cells, as indicated by ectopically expressed retinal neuronal markers. In contrast to the cyclopia phenotype caused by disrupting the *Shh* gene (Chiang *et al.,* 1996), reducing Shh signals after optic cup formation only result in the tissue identity switches of the ventral pigmented epithelium and the optic stalk. These findings are consistent with the recently established reciprocal regulatory relationship between the *Pax6* and *Pax2* genes (Schwartz *et al.,* 2000) and strongly indicate that Pax6 and Pax2 are critical for the specification of the retina and the optic stalk, respectively.

In addition to affecting *Pax2* and *Pax6* gene expression, altered Shh signals also affected the expression pattern of the *Otx2* gene, which has been implicated in development of the eye, including the pigmented epithelium (Kablar *et al.,* 1996; Bovolenta *et al.,* 1997; Bumsted and Barnstable, 2000). Misexpressing Shh causes ectopic *Otx2* expression in the peripheral retina, which subsequently becomes pigmented, whereas blocking endogenous Shh leads to a loss of *Otx2* expression in the ventral pigmented epithelium, which subsequently adopts a retinal fate. These results support a role of *Otx2* in specifying the pigmented epithelial fate in the optic cup. Previous studies have demonstrated that the retinal versus pigmented epithelial fate decision is regulated by FGFs (Park and Hollenberg, 1989; Guillemot and Cepko, 1992; Zhao *et al.,* 1997; Pittack *et al.,* 1997; Hyer *et al.,* 1998). Recent work has also revealed that factors in the extraocular mesenchyme, possibly TGFβ-like molecules, can influence the pigmented epithe-

lium specification of the early optic cup (Fuhrmann *et al.,* 2000). Therefore, it seems likely that Shh signals act in concert with other signaling molecules to pattern the pigmented epithelium by regulating the expression of *Otx2* and other critical genes.

Roles of Shh and BMP4 in D-V Pattern Formation of the Eye

It is well established that during neurulation, BMPs expressed in the ectoderm and dorsal neural tube are essential for determining the dorsal aspects of the central nervous system (Liem *et al.,* 1995, 1997; Tanabe and Jessell, 1996; Furuta *et al.,* 1997; Hogan, 1996). Among the BMP molecules expressed in the eye, BMP4 and BMP7 are known to be involved in early eye development (Dudley and Robertson, 1997; Dudley *et al.,* 1995; Luo *et al.,* 1995). In the mouse optic cup, *BMP4* expression is restricted to the dorsal retina, and disruption of the *BMP4* gene leads to defective lens formation and a possibly less developed dorsal optic cup (Furuta and Hogan, 1998). In chick, overexpression of *BMP4* caused ventral expansion of the dorsally expressed T-box transcription factor Tbx5, a dorsal retinal determinant (Koshiba-Takeuchi *et al.,* 2000) and reduction of the ventrally expressed *cVax* gene. Thus, BMP4 may serve to control dorsal properties of the retina in part through regulation of the transcription factor Tbx5.

The coordinated actions of Shh and BMP are required for multiple developmental systems (Heberlein and Moses, 1995; Tanabe and Jessell, 1996; Greenwood and Struhl, 1999; Vogt and Duboule, 1999). During chick optic primordium development, *Shh* and *BMP4* genes are expressed in non-overlapping opposing domains. We have demonstrated that misexpression of *Shh* at the optic vesicle stage abolished the dorsal expression of *BMP4* in the retina, indicating that high concentrations of Shh suppress *BMP4* transcription. Conversely, a reduction in Shh activity resulted in the ventral expansion of *BMP4* expression. These data suggest that Shh signal restricts the spatial distribution of BMP4 signals in the developing eye field by inhibiting the transcription of *BMP4.* Similar opposing regulatory relationships between Shh and BMP4 have been observed during organogenesis of multiple vertebrate organs and tissue types (Hirsinger *et al.,* 1997; Watanabe *et al.,* 1998; Schilling *et al.,* 1999; Capdevila *et al.,* 1999; Merino *et al.,* 1999; Zhang *et al.,* 2000). Thus, antagonistic regulation by Shh and BMP4 may serve as a common mechanism to establish and maintain distinct compartments originating from a single developmental field.

Consistent with the proposed roles for Shh and BMP4 in D-V patterning of the eye field, perturbing Shh levels in the chick optic vesicle also resulted in altered expression of the ventral determinant *cVax* (Schulte *et al.,* 1999). Similar to a previous study demonstrating that overexpression of *hedgehog* genes increases *Vax* gene expression domains in early *Xenopus* embryos (Hallonet *et al.,* 1999), we show an enhanced expression of *cVax* in the ventral retina by *Shh*
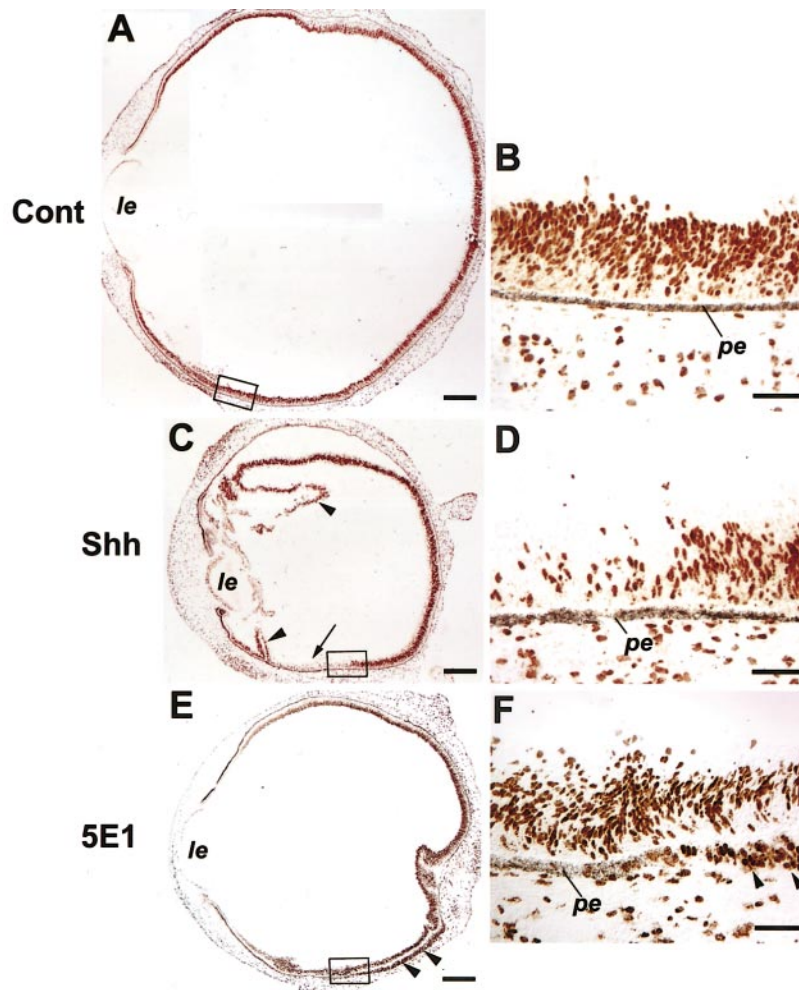
FIG. 9.   Effects of Shh signal levels on cell proliferation. Immunocytochemically staining for BrdU incorporation are shown. Control RCAS virus (A, B), Shh virus (C, D), and 5E1 hybridoma cell injections (E, F) were performed at stage 17, and eyes were labeled with BrdU for 3 h at stage 29 (E6). Control 3C2 hybridoma cell-injected eyes (not shown) show size and BrdU labeling patterns similar to those of control RCAS virus-infected eyes (A). Similar ventral regions (boxed A, C, E) near the optic fissure are also shown at higher magnification (B, D, F). Shh virus infection results in the folding of the peripheral retina (C, arrowheads) and fewer BrdU-positive cells in the ventral retina flanking the optic fissure (D, and arrow in C). 5E1 hybridoma cell injection leads to increased proliferation of the outer layer of the ventral optic cup, which has lost pigmentation (arrowheads in E and F). Scale bars in (A, C, E) represent 300 μm; scale bars in (B, D, F) represent 50 μm. le, lens; pe, pigmented epithelium.

overexpression and a reduced *cVax*-expressing domain in the eye as well as in the ventral brain by inhibiting Shh activity (X.-M. Zhang and X.-J. Yang, data not shown). Thus, *cVax* expression maybe directly or indirectly regulated by Shh signaling.

In chick, both the *cVax* and *Pax2* genes are expressed in the ventral optic primordium and induced by *Shh* misexpression. However, the onset of the anterior ventral expression of *cVax* occurs prior to the onset of *Pax2* expression in the ventral optic primordium. Later, *cVax* is expressed in a broader ventral domain (optic stalk and ventral retina) compared to the *Pax2* expression domain (optic stalk and ventral retina flanking the optic fissure) (X.-M. Zhang and X.-J. Yang, unpublished; Schulte *et al.,* 1999). In addition, forced expression of *Vax* genes results in the induction of *Pax2* gene expression (Barbieri *et al.,* 1999; Schulte *et al.,* 1999). In this study, we show that ectopic Shh expression resulted in augmented *cVax* expression limited to the ventral retina, which corresponded to the limited ventral expansion of the Pax2 domain. Taken together, these data suggest that *cVax* may act upstream of *Pax2* in determining ventral properties of the optic primordium.

In summary, proper Shh signals are critical for normal eye morphogenesis during and after the optic vesicle to optic
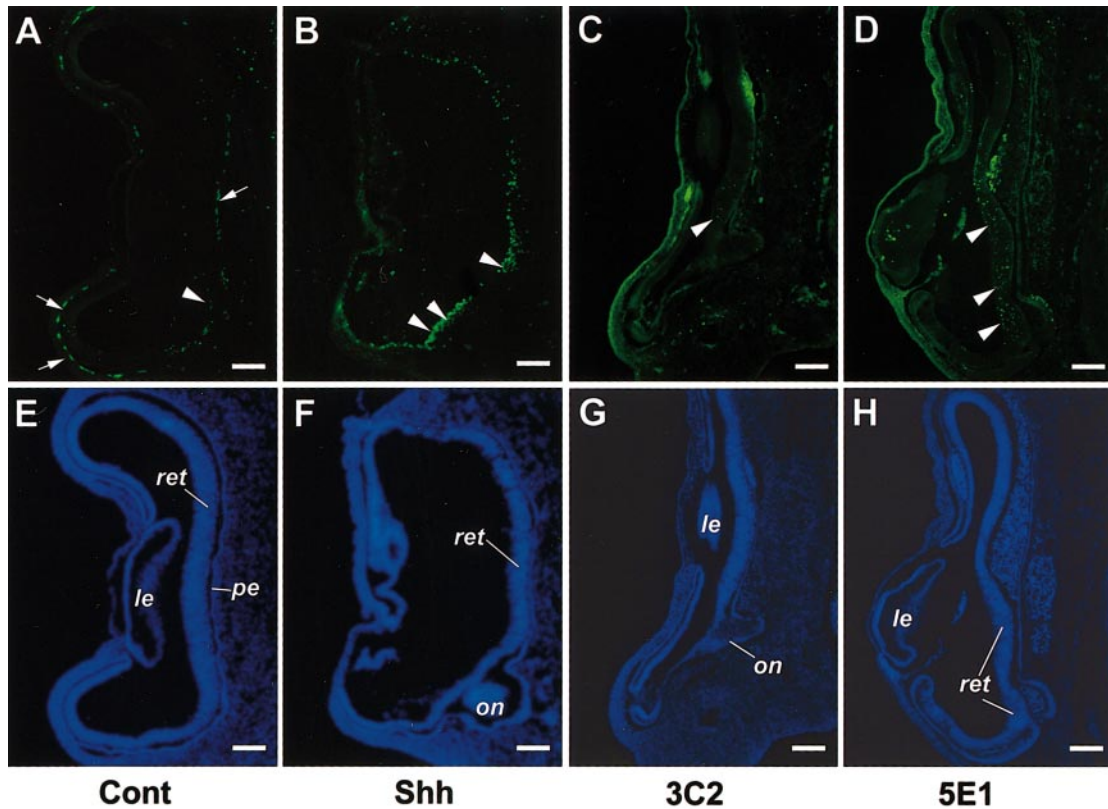
FIG. 10. Effects of altering Shh signal levels on cell death in the developing eye. The same sections of stage 24 eyes processed for TUNEL (A–D) and DAPI staining (E–H), respectively, are shown. The noninfected (A, E) and RCAS virus-infected eyes (data not shown) contain very few apoptotic cells located in the ventral retina near the optic nerve head (arrowhead, A). Shh virus infection (B, F) at stage 10 causes an increase of apoptotic cell death, especially in the ventral retina (arrowheads, B). Increased apoptosis is also observed in the pigmented epithelium layer of Shh virus-infected eyes (data not shown). Control 3C2 hybridoma cell-injected eyes show normal levels of cell death in the ventral retina at stage 24 (arrowhead, C). Eyes injected with 5E1 hybridoma cells at stage 17 (D, H) also contain increased apoptotic cells, which are distributed in the ventral retina (arrowheads, D). Arrows in (A) point to autofluorescent signals in surrounding tissues of the eye. Scale bars in all panels represent 100 μm. ret, retina; le, lens; pe, pigmented epithelium; on, optic nerve.

cup transition and for specification of different ocular tissues. The opposing Shh and BMP4 activities may be involved in establishing distinct dorsal and ventral eye compartments, as well as in promoting the growth and expansion of the entire eye field.

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