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The role of bone morphogenetic proteins in the differentiation of the ventral optic cup

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SUMMARY

The ventral region of the chick embryo optic cup undergoes a complex process of differentiation leading to the formation of four different structures: the neural retina, the retinal pigment epithelium (RPE), the optic disk/optic stalk, and the pecten oculi. Signaling molecules such as retinoic acid and sonic hedgehog have been implicated in the regulation of these phenomena. We have now investigated whether the bone morphogenetic proteins (BMPs) also regulate ventral optic cup development. Loss-of-function experiments were carried out in chick embryos in ovo, by intraocular overexpression of noggin, a protein that binds several BMPs and prevents their interactions with their cognate cell surface receptors. At optic vesicle stages of development, this treatment resulted in microphthalmia with concomitant disruption of the developing neural retina, RPE and lens. At optic cup stages, however, noggin overexpression caused colobomas, pecten agenesis, replacement of the ventral RPE by neuroepithelium-like tissue, and ectopic expression of optic stalk markers in the region of the ventral retina and RPE. This was frequently accompanied by abnormal growth of ganglion cell axons, which failed to enter the optic nerve. The data suggest that endogenous BMPs have significant effects on the development of ventral optic cup structures.

Key words: BMPs, Retina, Chick, Optic stalk, Differentiation, Chick

INTRODUCTION

Dorsal and ventral retinae differ in many respects, from the topographically specific projection of ganglion cell axons, to regional differences in cell surface molecules (reviewed by Goodhill and Richards, 1999; O'Leary and Wilkinson, 1999). They diverge at early embryonic stages, when the developing optic cup expands faster dorsally than ventrally, while the choroid fissure forms in the ventral retina (Koshiba-Takeuchi et al., 2000). Discrete dorsal and ventral optic cup compartments are apparent in the differential expression of transcription factors (Macdonald et al., 1995; Torres et al., 1996; Barbieri et al., 1999; Ohsaki et al., 1999; Schulte et al., 1999; Koshiba-Takeuchi et al., 2000), and in their regulation by signaling molecules (Marsh-Armstrong et al., 1994; Hyatt et al., 1996; Koshiba-Takeuchi et al., 2000; Zhang and Yang, 2001). Regions of high and low retinoic acid concentration (Mey et al., 1997; Mey et al., 2001; McCaffery et al., 1999), and the initial domains of expression of mRNA for BMP2 and BMP7 (Belecky-Adams and Adler, 2001), also define dorsoventral molecular boundaries.

The ventral region of the chick embryo optic cup undergoes additional specialization into four structures: the neural retina, the retinal pigment epithelium (RPE), the optic disk/optic stalk, and the pecten oculi, which protrudes into the vitreous and provides blood supply to the otherwise avascular retina. These adjacent structures become separated by sharp functional, molecular and/or structural boundaries. For example, mesenchymal cells invade the optic cup through the choroid fissure, and colonize the rudimentary pecten, but do not invade the retina proper (reviewed by Wolberg et al., 1999). Retinal ganglion cell axons grow along the vitreal surface of the retina towards the optic stalk, where they abandon the retina, avoid the RPE and pecten, and turn into the optic stalk (which thus becomes optic nerve). An example of molecular boundaries is the reciprocal exclusion of PAX2 and PAX6, with PAX2 being restricted to the optic stalk/optic disk area and PAX6 to the neural retina and early RPE (Nornes et al., 1990; Li et al., 1994; Schwarz et al., 2000). Differentially expressed cell adhesion and axonal guidance molecules include netrin, localized to the optic stalk region, R-cadherin, limited to the optic nerve head and pigmented epithelium, and B-cadherin, found in the pecten (Redies et al., 1993; Wohrn et al., 1998; Gerhardt et al., 2000).

Several signaling molecules have been implicated in the control of ventral optic cup development. Sonic hedgehog (SHH) overexpression at early stages of chick retina development, for example, resulted in ciliary margin defects, a reduction of the ventral retina, and optic nerve hyperplasia; inhibition of SHH signaling brought about the development of a secondary retina in place of the ventral RPE (Zhang and Yang, 2001). In other species, retinoic acid (RA) administration caused duplications of the ventral retina and/or ventral eye structures (Hyatt et al., 1992; Hyatt et al., 1996), while experimental interference with retinoic acid signaling

triggered ventral retina losses (Marsh-Armstrong et al., 1994). RA-synthesizing enzymes are differentially distributed within the retina in several species including the chick (McCaffrey et al., 1991; McCaffrey et al., 1992; McCaffrey et al., 1993; Mey et al., 1997; Grun et al., 2000; Mic et al., 2000; Suzuki et al., 2000). The studies reported here focused on yet another group of factors, the bone morphogenetic proteins (BMPs).

The BMPs have many actions in the nervous system, including patterning, cell fate determination, apoptosis, and cell proliferation (Mehler et al., 1997). Their activities are modulated by extracellular binding proteins, including noggin, chordin, cerberus and follistatin. These proteins bind one or more of the BMPs, preventing their interactions with BMP receptors (reviewed by Smith, 1999). It has been suggested that BMP4 is involved in the dorsalization of the retina (Koshiba-Takeuchi, 2000), and that the recently identified BMP4 antagonist ventroptin prevents it from affecting the ventral retina (Sakuta, 2001). A related finding was that SHH, which regulates ventral retina development, causes the downregulation of dorsally expressed BMP4 (Zhang and Yang, 2001). Taken together, these observations appear to suggest that active BMP4 is prevented from reaching ventral eye tissues form during normal development, thus excluding it as a putative regulator of the differentiation of optic stalk, pecten, ventral retina and ventral RPE. We have observed by in situ hybridization, however, that at early stages of development the BMP receptors are found primarily, if not exclusively, in the *ventral* portion of the retina and in the optic stalk (Belecky-Adams, 2001), suggesting that these tissues could be affected directly by the BMPs.

In the present study, we have shown that the BMPs indeed have profound effects on the developing ventral retina. Disruption of BMP signaling by overexpressing the BMP binding protein noggin resulted in microphthalmia at early stages of development. When performed at somewhat later stages, however, noggin overexpression resulted in a host of changes in the ventral eye, including coloboma formation, dysmorphogenesis of the optic stalk region, transdifferentiation of a portion of the ventral RPE into optic stalk-like tissue, and abnormalities in lens differentiation. A more detailed investigation of colobomatous eyes showed changes in the pattern of expression of dorsoventral markers, and an expansion into the putative retina of the territory of expression of optic stalk molecular markers. Many of these changes appeared similar to those triggered by SHH overexpression (Nasrallah and Golden, 2001; Zhang and Yang, 2001), suggesting that both BMPs and SHH participate in the development of the ventral retina.

MATERIALS AND METHODS

Materials

Antibodies against AMV-3C2, Pax6, 7G4, 40.2D6 (Developmental Hybridoma Bank, Iowa City, IA); normal goat serum, Brn3a antibody (Chemicon, Temecula, CA); GenePorter™ (Gene Therapy Systems, San Diego, CA); GFP expression vector, RNAse A, formamide, tRNA, restriction enzymes, DNA purification kit, DMEM, fetal calf serum, chick serum (In Vitrogen, Carlsbad, CA); hydrogen peroxide, methanol, dimethylsulfoxide, sodium citrate (JT Baker, Phillipsburg, NJ); Alexa Fluor-labeled secondary antibodies (Molecular Probes, Eugene, OR); aquamount (Polysciences, Warrington, PA); blocking buffer, anti-digoxigenin FAB fragments, RNAse T1, RNA polymerases T3, T7, SP6, digoxigenin-labeled NTPs, 5-bromo-3-indolyl-phosphate (BCIP), nitro blue tetrazolium chloride (NBT) (Roche, Indianapolis, IN); Triton X-100, sucrose, Chaps, paraformaldehyde, picric acid, Tween 20, heparin (Sigma, St Louis, MO); P27 anti-retroviral antibody (SPAFAS, North Franklin, CT); ABC elite vectastain, biotinylated secondary IgGs (VectorLabs, Burlingame, CA).

Line 0 pathogen-free white leghorn eggs were obtained from B and E eggs (Stevens, PA).

Methods

Retroviral production

Replication competent RCAS BP (A) retroviruses (Hughes et al., 1987) engineered to express noggin, dominant negative BMP receptor 3, or alkaline phosphatase, were generous gifts from Cliff Tabin (Harvard; Boston, MA), Tsutomu Nohno (Kawasaki Medical School; Kurashiki City, Okayama, Japan), and Constance Cepko (Harvard; Boston, MA), respectively. Retroviral stocks were prepared as described previously (Morgan and Fekete, 1996); a minor modification was that cultured embryonic fibroblasts from Line 0 pathogen-free chickens were co-transfected by mixing with the RCAS vector of interest and 10% volume of a green fluorescent protein-expression vector with GenePorter™, as directed by the manufacturer.

Microinjection

Glass micropipettes were pulled using a Flaming Brown pipette puller (Sutter Instrument Co., Novato, CA), beveled with a BV-10 microelectrode beveler (Sutter Instrument Co.) at 25° angle to 12-18 μm inner diameter, sterilized by UV irradiation, and rinsed with sterile BSA-containing LEBM prior to injection. Eggs were opened according to the method of Selleck (Selleck, 1996). The right eye of chock embryos (Hamburger and Hamilton stages 8-10, or 15-18) was penetrated dorsally with the micropipette, and injected intravitreally with 0.5-1.0 μl of 5×10^8–1×10^9 CFUs/ml concentrated retroviral stock, using a PLI-90 picoliter injector (Harvard Apparatus, Inc. Holliston, MA) for 1-2 mseconds at 18 psi. Egg windows were closed with transpore tape, and returned to the incubator; they were re-injected 3 hour later with the same amount of retroviral stock.

Immunocytochemistry

Antibodies, generously donated by the noted investigators, were diluted as follows. Anti-visinin polyclonal (Thierry Leveillard, Universite Louis Pasteur, Strasbourg, France) 1:1000; anti-netrin polyclonals PN2 and PN3 (Tim Kennedy, McGill University, Montreal, Quebec, Canada) (Mannitt et al., 2001), 1:500 and 1:1000, respectively; MC/1 (anti-MMP115; Makoto Mochhi, Himeji Institute of Technology, Hyogo, Japan) (Mochii et al., 1988) 1:250; RCD2 (anti-R-cadherin from M. Takeichi supplied by Jerry Grunwald, Jefferson Medical College, Philadelphia, PA) (Wohrn et al., 1998), undiluted; and anti-PROX1 (Slava Tomarev, National Eye Inst., NIH, Bethesda, MD) (Belecky-Adams et al., 1997), 1:500. Commercial antibodies (see Materials) were AMV 3C2 (1:10), 7G4 (anti-visinin monoclonal, 1:10), BRN3A (1:100), 40.2D6 (islet 1, 1:35), PAX6 (1:5), and PAX2 (1:200). Immunocytochemistry was generally carried out as in Belecky-Adams et al. (Belecky-Adams et al., 1996). Embryos processed for netrin immunocytochemistry were fixed with 4% paraformaldehyde, 15% picric acid in PBS, pH 8.0, cryosectioned, microwaved twice in 10 mM sodium citrate buffer pH 6.0 for 10 minutes on high setting, and cooled in PBS prior to blocking in goat serum.

In situ hybridization

The following probes were generously provided by these investigators: ALDH1 and ALDH6, by Felix Grun (University of California, Irvine, CA); VAX, by Constance Cepko (Harvard Medical

School, Boston, MA); netrin 1, by Marc Tessier-Levigne (University of California, San Francisco, CA); TBX5, by Katherine Yutzey (Children's Hosptial Medical Center, Cincinnati, OH); FGF8, by Gail Martin (University of California, San Francisco, CA); PAX2, by R.-M. Alvarado-Mallart (Hospital de la Salpetriere, Paris, FR); and SHH and patched, by Cliff Tabin (Harvard Medical School, Boston, MA). Probes were labeled as in Belecky-Adams and Adler (Belecky-Adams and Adler, 2001). For in situ hybridization, embryos were embedded in sucrose/OCT as described in the Immunocytochemistry section, sectioned at 10-12 μm, rinsed with PBS containing active DepC for 10 minutes at room temp prior to incubating in hybridization buffer, and processed as described previously (Belecky-Adams et al., 1997).

RESULTS

The goal of these experiments was to investigate the role of BMPs in the developing retina, using a retroviral vector to overexpress the BMP-binding protein, noggin. Noggin is a secreted protein that binds homo and heterodimers containing BMP2 or BMP4 with high affinity, and BMP7 with lower affinity, thus preventing their interactions with cell surface receptors (reviewed by Mehler et al., 1997). Injections of RCAS retroviruses coding for either noggin or the control, alkaline phosphatase, were done at optic vesicle stages (H and H stage 9-10), or after optic cup formation (H and H stage 15-18). Expression of retroviral antigens became detectable 9-11 hours after injection (data not shown). Injected embryos were analyzed on E6 or E8.

Microinjection of noggin retrovirus at optic vesicle stages led to microphthalmia

Eyes injected with the control alkaline phosphatase retrovirus prior to formation of the optic cup showed no detectable histological changes as compared to untreated controls (Fig. 1A), whereas the majority (9/12) of eyes receiving the noggin retrovirus were clearly microphthalmic (Fig. 1B). Histologically, the microphthalmic eyes appeared to have a much smaller lens (arrow, Fig. 1D), and a bilayered structure that resembled (but was not identical to) an optic cup with optic stalk, retina and retinal pigment epithelium (arrowhead, Fig. 1E). Additional abnormalities included lack of vitreous chamber, apparent expansion of the ventricular cavity, disorganization of the periocular mesenchyme, and ectopic localization of mesenchyme-like tissue around the lens (Fig. 1D,E).

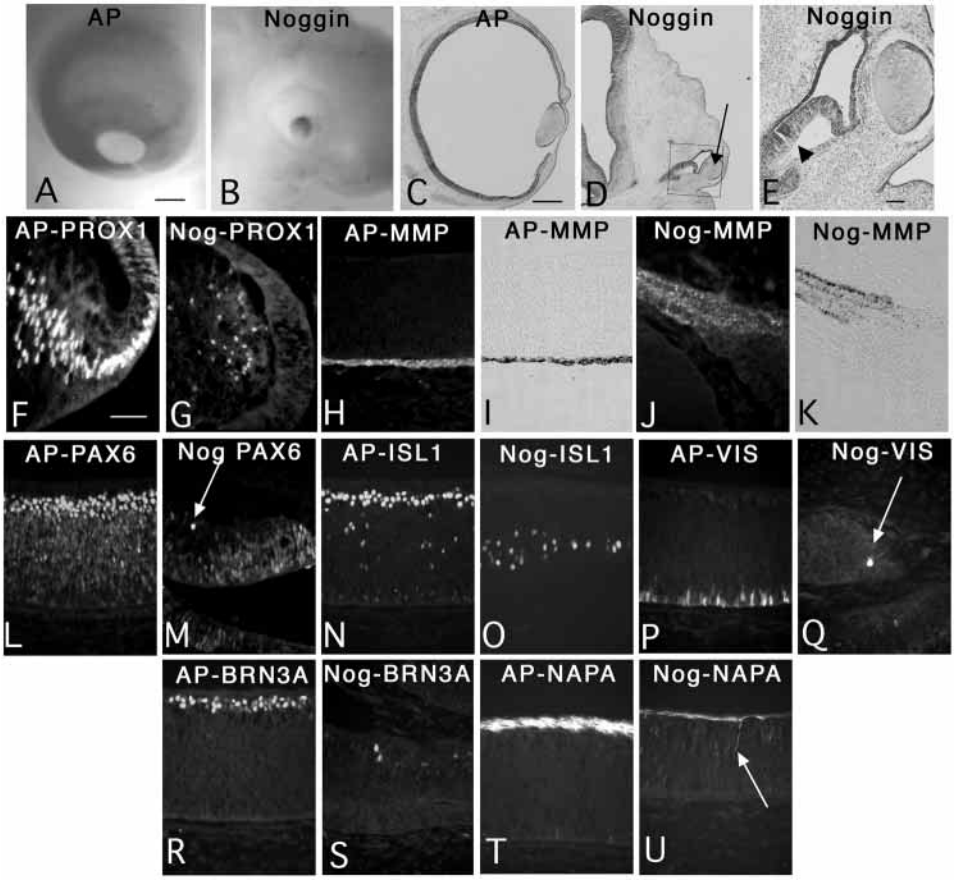

Fig. 1. Lack of BMP signaling leads to microphthalmia. Embryos were infected at HH stage 9-11 with retrovirus expressing either alkaline phosphatase (AP; A,C,F,H,I,L,N,P,R,T) or the BMP-binding protein noggin (B,D,E,G,J,K,M,O,Q,S,U), and studied at E6. (A-E) Gross morphology. Noggin-infected eyes (B) appeared much smaller and less pigmented than alkaline phosphatase controls (A). In histological sections, alkaline phosphatase-infected controls (C) showed no gross abnormalities, while noggin-infected eyes contained rudimentary lens (D, arrow) and neural retina, pigment epithelium, and optic stalk (E, arrowhead). C and D are the same magnification. (F,G) Expression of PROX1 in the lens. Few irregularly distributed PROX1-positive nuclei are scattered throughout the lens of noggin-infected eyes (G), but are more abundant and regularly arranged in alkaline phosphatase-infected controls (F). (H-K) Expression of the pigment-epithelial marker MMP115. Despite the abnormal configuration of the eye in noggin-infected embryos, some pigment epithelium differentiation was apparent both in the presence of pigmented cells (K) and immunoreactivity for MMP115 (J). However, these cells appear less well organized than their counterparts in alkaline phosphatase-infected controls (H,I). (L-U) Expression of neural retina-specific markers. Retinal-specific markers, such as PAX6, islet 1, visinin, and BRN3A, were found in both alkaline phosphatase- (L,N,P,R) and noggin-infected eyes (M,O,Q,S). Positive cells were scarcer and less regularly distributed in noggin-infected eyes. (T,U) Ganglion cell axons. Fibers immunoreactive with the NAPA73 antibody, which is specific for ganglion cell axons, appeared less abundant in noggin-infected eyes, in which they followed abnormal trajectories into the neuroepithelium (arrow U). AP, alkaline phosphatase-infected; Nog, noggin-infected; MMP, MMP115, ISL1; islet 1; VIS, visinin; NAPA, NAPA73. Scale bars: A,B, 50 μm; C,D, 500 μm; E, 50 μm; F-U, 50 μm.

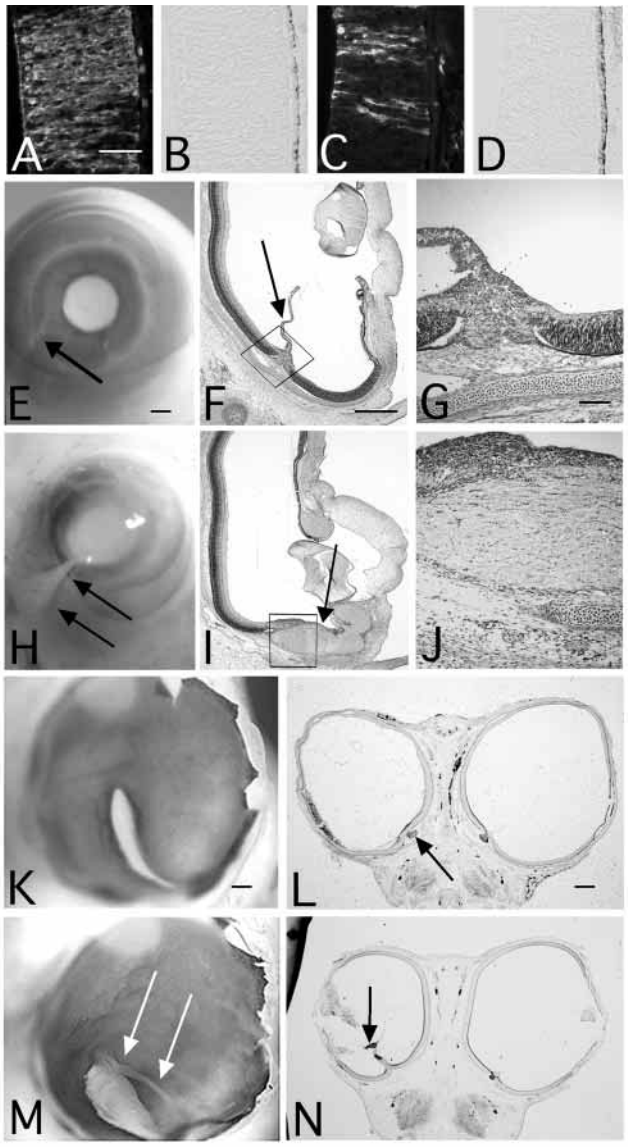

Fig. 2. Ventral eye abnormalities in embryos injected with RCAS-noggin at HH stages 15-18. (A-D) Expression of viral gag proteins in eyes infected with RCAS-alkaline phosphatase or RCAS-noggin. Immunocytochemistry with AMV 3C2 antibody, which recognizes retroviral antigens, was carried out to monitor the extent of infection within each treated eye. Sections through eyes infected with alkaline phosphatase (A,B) or noggin (C,D) retrovirus showed infection throughout the retina, indicating that both retroviral stocks contained infective viral capsids. (E-J) Anatomical and histological features. E8, alkaline phosphatase controls (E-G) show a normally closed choroid fissure in the anterior segment of the eye (arrow, E), normal retina (F), and the presence of the pecten, depicted at both low (F, arrow) and high magnification (G, corresponding to boxed area in F). (H-J) Noggin-infected eyes showed lack of choroid fissure closure (colobomas; H, arrows). The pecten was undetectable in histological sections, and a large mass of mesenchymal-like tissue was noted in the ventral region of the optic cup (I, arrow; J is a higher magnification of the boxed region from I). (K,M) Internal aspect of the fundal region of eyes infected with alkaline phosphatase (K) or noggin (M), as seen after dissecting away the anterior region of the eye. An enlarged choroid fissure was found in noggin-infected embryos in the posterior chamber (M). The fissure was still present, but diminished, in the posterior pole of alkaline phosphatase controls (K). Noggin-infected embryos also showed a white fibrous material, projecting into the vitreal cavity of the eye (arrows, M). (L,N) Sections through the fundal region of alkaline phosphatase- (L) and noggin-infected (N) eyes, labeled with antibodies specific for class III β-tubulin. Controls show darkly labeled ganglion cell axons at the vitreal edge of the retina, and into the optic nerve head (arrow, L). In noggin-infected eyes, in contrast, fibers were generally undetectable in the optic nerve but could be seen projecting into the vitreal chamber (arrow, N), corresponding to those in M. Scale bars: A-D, 50 μm; E,H, 500 μm; F,I, 500 μm; G,J, 50 μm; K,M, 500 μm; L,N, 500 μm.

Tissues present in microphthalmic eyes were further characterized using antibodies against lens, RPE and neural retina markers. In controls, the transcription factor PROX1 showed intense immunoreactivity in the nuclei of elongating lens fibers, and weaker signal in the anterior lens epithelium, (Fig. 1F), while noggin-infected lenses showed fewer PROX1-positive nuclei (Fig. 1G), which appeared irregularly distributed. An antibody against RPE-specific protein, MMP115, showed positive cells in both the control (Fig. 1H,I) and noggin-infected embryos (Fig. 1J,K). The MMP115-positive RPE abutted the neural retina in untreated and alkaline phosphatase-infected eyes, however, it appeared to be highly irregular and frequently distant from the neuroepithelium in noggin-infected embryos (Fig. 1D,E). When compared with alkaline phosphatase-infected retinas (Fig. 1L,N), noggin-infected embryos immunostained for PAX6 (Fig. 1M) or islet 1 (Fig. 1O) showed fewer positive cells, which were no longer found in the laminar pattern of distribution seen in controls. Cells immunoreactive for visinin (a photoreceptor-specific marker) and BRN3A (a ganglion-cell specific marker) were extremely sparse in noggin-treated eyes (compare Fig. 1P,R with Q,S). In the latter, NAPA73-positive ganglion cell axons appeared less intense than in controls, and followed abnormal trajectories away from the vitreal surface of the retina (Fig. 1T,U).

Microinjection of noggin retrovirus after optic cup formation led to complex abnormalities of the ventral eye

Experiments described below involved embryos injected between H and H stages 15-18, and terminated on E6 or E8. The extent of infection was evaluated immunocytochemically with anti-viral antibodies; effective expression of viral antigens was observed in sections of both alkaline phosphatase- and noggin-injected eyes (Fig. 2A-D). Many phenotypic changes observed in noggin-infected eyes involved the choroid fissure, a ventral landmark that extends from the anterior edge of the optic cup to the optic stalk (Fig. 2E,H). The choroid fissure appeared completely closed in all the alkaline phosphatase-injected eyes (Fig. 2E), as in untreated controls (not shown). However, 79% of noggin-injected embryos (89/113) had a coloboma, reflecting incomplete closure of the choroid fissure. Colobomas were already evident at E6 (not shown), and became even more conspicuous at E8 (Fig. 2H), when the optic fissure was clearly closed in the anterior segment of the controls (Fig. 2E).

Histologically, noggin-infected eyes showed a mass of

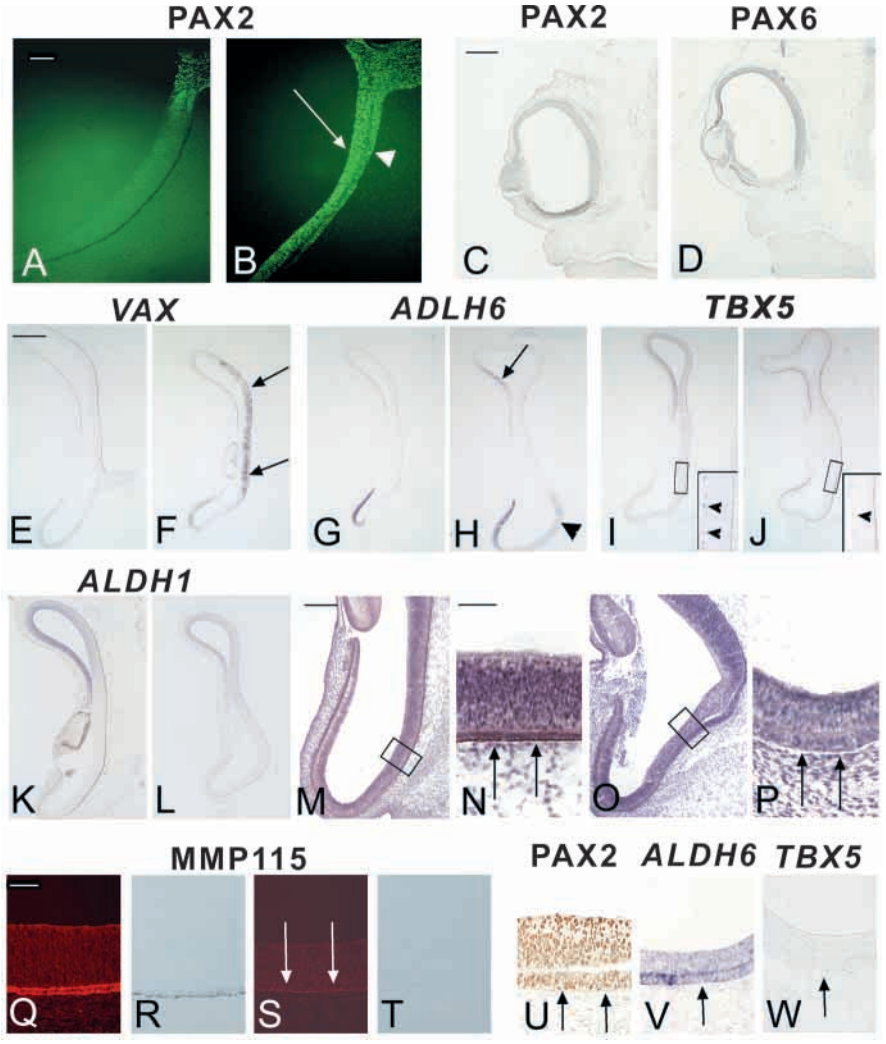

Fig. 3. Expression of dorsal and ventral markers. Embryos were infected on E3 (HH 15-18) and analyzed on E6. (A-D) PAX2 and PAX6. PAX2 appeared restricted to the optic stalk in control eyes (A), but in noggin-infected embryos (B) it was detectable in the optic stalk, ventral retina (arrow) and a secondary neuroepithelium that replaced the RPE (arrowhead). The region of PAX2 expression in the ventral retina of noggin-infected eyes (C) was completely devoid of PAX6 expression (D), consistent with reports from the literature (Schwarz et al., 2000). (E-H) Expression of ventral retinal markers. *VAX* expression appeared weak and restricted to the ventral portion of alkaline phosphatase-infected retinas (E), but was much stronger and encompassed both ventral and dorsal regions of the retina in noggin-treated eyes (F). *ALDH6* was restricted to the ventral region of the presumptive ciliary body in alkaline phosphatase controls (G), but was also found in the presumptive dorsal ciliary body (arrow, H) and throughout the ventral retina in noggin-infected eyes (arrowhead, H). (I-L) Expression of dorsal retina markers. Two dorsal markers, *TBX5* and *ALDH1*, appeared much more intense in alkaline phosphatase controls (I,K), than in noggin-treated retinas (J,L). *TBX5* expression was seen in a subset of ventral ganglion cells (arrowheads) in both control and noggin-treated eyes (boxes in I and J). (M-W) Changes in ventral pigment epithelium. Noggin-infected eyes appeared to be missing the ventral pigment epithelium in the region adjacent to the coloboma (O,P). The region that normally differentiates as ventral RPE in control eyes (arrows, N) was replaced by ectopic neuroepithelium-like tissue in noggin-treated eyes (arrows; P). This secondary neuroepithelial-like structure did not express pigment or the RPE-specific protein MMP115 (arrows, S,T), which are detectable in alkaline phosphatase controls (Q,R), but it was positive for two ventral retina/optic stalk markers, PAX2 and *ALDH6* (arrows, U,V). None of these structures was positive for dorsal retina markers such as *TBX5* (arrow; W). ALDH6, aldehyde dehrdrogenase 6; ALDH1, aldehyde dehydrogenase 1. Scale bars: A,B, 500 μm; C,D, 500 μm; E-L, 500 μm; N,P, 50 μm; M,O, 50 μm; Q-W, 50 μm.

mesenchymal-like tissue in the region of the coloboma occupied by ventral retina in normal embryos at E8 (arrow, Fig. 2I). The pecten, which normally protrudes into the vitreous chamber (arrow, Fig. 2F), appeared to be altogether missing in many noggin-treated embryos (Fig. 2I,J). The optic nerve head was displaced towards the ventral periphery in noggin-infected eyes (compare Fig. 2K,M). Finally, in a small percentage (9%, 8/89; Fig. 2M) of the noggin-infected eyes with colobomas, there was dysmorphogenesis of the optic nerve, including ectopic nerve fibers projecting into the vitreous in the region normally occupied by the pecten. These fibers were immunoreactive for RA4, NAPA73 and β-tubulin class III (as illustrated for β-tubulin III in Fig. 2L,N), suggesting that they were of ganglion cell origin (Snow and Robson, 1994; Waid and McLoon, 1995; Austin et al., 1995).

Overexpression of noggin results in changes in the distribution of dorsoventral markers

Sections of E6 embryos were immunoreacted for dorsal or ventral retinal markers to investigate possible changes in dorsoventral polarity. PAX2 was restricted to optic nerve head and pecten in alkaline phosphatase-injected eyes (Fig. 3A), as in uninjected embryos (not shown). In contrast, the PAX2-positive domain was much broader in noggin-injected eyes, extending from the optic nerve head into the ventral retina (arrow, Fig. 3B). Ectopic ventral retinal expression of PAX2 (Fig. 3C) was accompanied by the disappearance of PAX6 from that region (Fig. 3D), where it is normally found in control embryos (not shown). This is in agreement with reports that PAX2 and PAX6 are mutually exclusive (Schwarz et al., 2000). Noggin-injected embryos had a second PAX2 expression domain, in neuroepithelial tissue adjacent to the ventral neural retina, roughly corresponding to the position normally occupied by the pigmented epithelium (arrowhead, Fig. 3B, and see below).

As previously reported (Schulte et al., 1999) *VAX* showed a ventral domain of expression in controls (Fig. 3E), whereas *VAX* signals appeared stronger in the ventral retina, and extended into

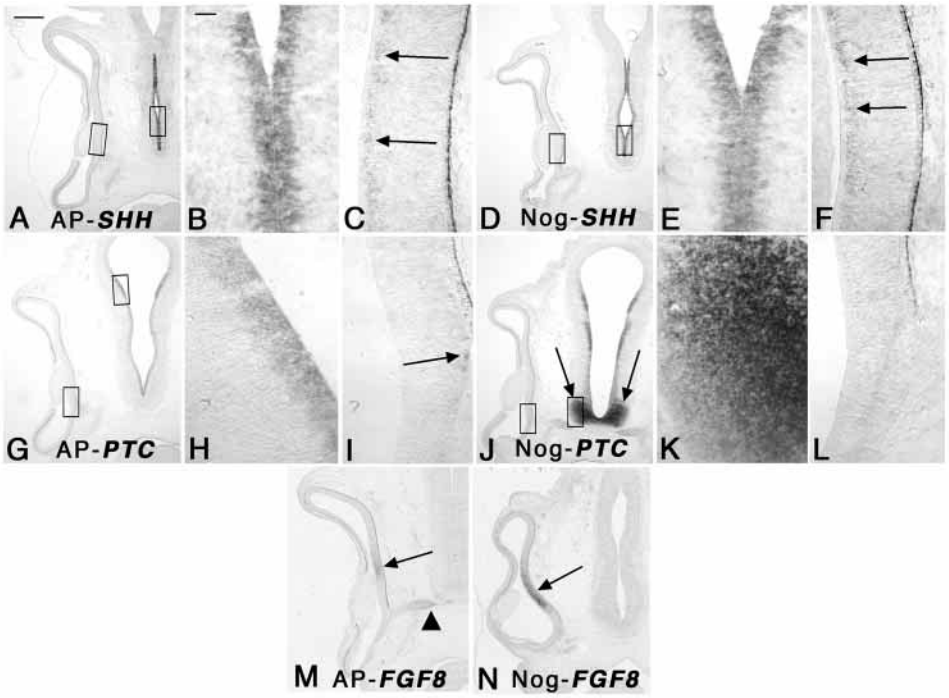

Fig. 4. Expression of *SHH* and *FGF8* in noggin-infected and control eyes. Embryos were infected at stage 15-18 with RCAS-noggin or RCAS-alkaline phosphatase and were analyzed on E6. (A-F) *SHH* expression. Both alkaline phosphatase- and noggin-infected eyes expressed *SHH* very heavily in the ventral neural tube (A,B and D,E), and weakly in the neural retina (arrows, C,F). No apparent changes in either the relative intensity or pattern of distribution of the signals were noted in noggin-infected eyes (D-F) in comparison to controls (A-C). (G-L) *PTC* expression. Intensity of signals for the transcripts of the SHH receptor patched (*PTC*) provides an indication of levels of SHH signaling because *PTC* is upregulated by SHH (Marigo and Tabin, 1996; Goodrich et al., 1996). *PTC* expression was conspicuous in the neural tube (G; top box enlarged in H); there was very little expression in the neural retina, restricted to the optic nerve head region (G; lower box enlarged in I; arrow points to positive cells). Embryos infected with noggin showed increases in signal in the neural tube (arrows in J; box enlarged in K), likely due to seepage of intravitreally injected virus into the neural tube through the optic stalk. No such increases in *PTC* signal were observed in the retinae of the same noggin-infected embryos (J, box enlarged in L). (M,N) *FGF8* expression. *FGF8* was weakly expressed in a region just dorsal to the optic stalk in alkaline phosphatase-infected controls (arrow, M), as well as the optic stalk itself (arrowhead, M). In noggin-infected eyes, however, *FGF8* signal was much more intense and found in a larger region than in controls (arrow, N). Scale bars: A,D,G,J,M,N, 500 μm; B,C,E,F,H,I,K,L, 50 μm.

the dorsal retina in noggin-treated eyes (arrows, Fig. 3F). Transcripts for the retinoic acid-synthesizing enzyme aldehyde dehydrogenase 6 (ALDH6) were restricted to the presumptive ventral ciliary body in alkaline phosphatase-infected embryos (Fig. 3G), but in noggin-infected eyes it expanded both to the dorsal region of the presumptive ciliary body (arrow, Fig. 3H) and to the ventral retina (arrowhead, Fig. 3H). Signals for two dorsal retina markers, *TBX5* and *aldehyde dehydrogenase 1* (*ALDH1)*, appeared to be somewhat less intense but not completely abolished in noggin-infected eyes (Fig. 3I-L).

Lack of BMP signaling results in expansion of the optic stalk at the expense of the pigmented epithelium

A two- to three-cell-layer thick, PAX2-positive neuroepithelium-like structure was noted adjacent to the ventral region of the neural retina in noggin-infected eyes (see above, and Fig. 3A,B and O,P). This tissue was negative for the RPE marker MMP115 (compare Fig. 3Q with 3S), but expressed several markers normally found in the ventral retina and/or in the optic stalk, including PAX2 (Fig. 3U), *ALDH6* (Fig. 3V), *FGF8*, and *VAX* (not shown). Dorsal retina was lacking markers such as *TBX5* (Fig. 3W) or *ALDH1*, as well as differentiated retinal cell markers which are normally widespread throughout the retina, including islet 1, visinin, and BRN3A (data not shown).

Lack of BMP signaling results in an increase in FGF8 expression

The above-mentioned secondary neuroepithelium was reminiscent of the transdifferentiation of RPE cells into neural retina caused by *FGF8* treatment of the chick embryo eye (Vogel-Hopker et al., 2000). We investigated, therefore, whether noggin had any effects on *FGF8* expression. Control embryos showed *FGF8* signals in the optic nerve and adjacent retina (Fig. 4M) (see also Vogel-Hopker et al., 2000). Noggin-infected eyes, in contrast, showed both increased signal intensity and a dorsally expanded expression domain (Fig. 4N, arrow). The ventral retina remained devoid of *FGF8* signal, but the secondary neuroepithelium itself was *FGF8* positive in noggin-treated embryos (not shown), resembling the situation in FGF8-induced transdifferentiation (Vogel-Hopker et al., 2000).

Changes in ventral retina were not accompanied by changes in expression patterns of sonic hedgehog or patched

Sonic hedgehog (*SHH*), which plays an important role in the development of the ventral retina (Zhang and Yang, 2001), was abundant in the ventral region of the forebrain (Fig. 4A,B,D,E), but was weakly expressed in the retinas of both control and noggin infected eyes (Fig. 4C,F). Transcripts for the SHH receptor patched (*PTC*), which is upregulated in response to increased SHH signaling (Marigo and Tabin, 1996; Goodrich et al., 1996), did indeed appear more abundant and widespread in the ventral forebrain of noggin-injected embryos (compare Fig. 4J,K with G,H) but there were no obvious differences in *PTC* distribution or signal intensity between the retinas of noggin- and alkaline phosphatase-treated retinas (Fig. 4I,L).

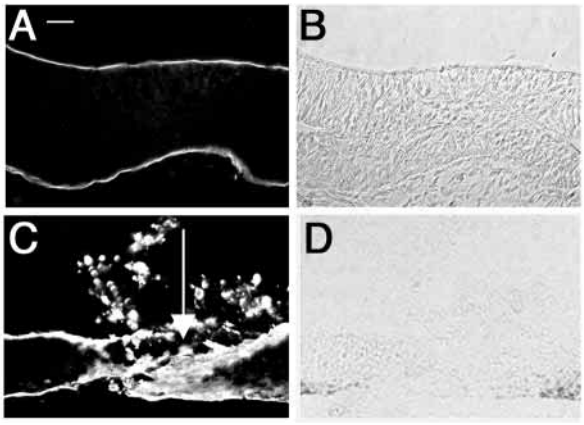

Fig. 5. Laminin immunoreactivity in colobomatous eyes. Embryos infected at stage 15-18 (E3) were processed immunohistochemically at stage 29 (E6) with antibodies against laminin. (A,B) Alkaline phosphatase-infected eye. In the area where the choroid fissure is closing, laminin immunoreactivity is present only at the vitreal edge of the retina (top) and at the choroidal surface of the pigmented epithelium (bottom). (C,D) Noggin-infected retinas. The choroid fissure has not closed in an area similar to that depicted in A, B. Laminin immunoreactivity is observed in the region between the two opposing surfaces of the retina and RPE in this area (arrow, C). Scale bars: 50 μm.

Lack of BMP signaling results in changes in laminin distribution

A lack of basal lamina degradation has been reported in colobomatous eyes (Hero, 1989; Hero, 1990; Hero et al., 1991; Torres et al., 1996), and may be causally related to this defect (Torres et al., 1996). A comparison of sections from alkaline phosphatase- and noggin-infected retinas immunostained with laminin-specific antibody showed that laminin was undetectable in the region of the normally closed choroid fissure in control embryos (Fig. 5A,B), but was still conspicuously present in noggin-infected eyes (Fig. 5C,D).

Optic nerve dysmorphogenesis is accompanied by changes in the expression patterns of R-cadherin and netrin

Some noggin-infected embryos have severe optic nerve abnormalities (see above). Several molecules that are known or suspected to regulate and/or guide ganglion cell axonal growth, and are broadly distributed throughout the retina, were indistinguishable in alkaline phosphatase- and noggin-infected eyes. They include agrin (Fig. 6A,B), chondroitin sulfate proteoglycan (Fig. 6C,D), and nidogen and collagen-14 (not shown). R-cadherin and netrin, however, showed striking differences between treatments. As previously reported (Gerhardt et al., 2000), in control embryos there was a sharp boundary between an R-cadherin-positive domain, which included the optic disk and the immediately adjacent retina, and the rest of the retina, which appeared R-cadherin negative (Fig. 6E,F). In noggin-infected eyes, R-cadherin immunoreactivity was seen in a much broader domain extending into the ventral retina and the region of the coloboma (Fig. 6G,H). Netrin immunoreactivity also predominated in the optic nerve and optic disk in control embryos, with additional staining in the ventral aspect of the presumptive ciliary body (Fig. 6I-L). In contrast, netrin immunoreactivity was conspicuous throughout the ventral retina of noggin-infected eyes (Fig. 6M-P). In situ hybridization analysis of alkaline phosphatase controls detected *netrin1* mRNA in the optic nerve (Fig. 6Q) and presumptive ciliary body (not shown), but not in ventral retina or RPE (Fig. 6R). In contrast, noggin-infected eyes showed expression in the optic nerve (Fig. 6S), in the secondary neuroepithelium that developed adjacent to the colobomatous area (Fig. 6T), and in the ciliary body (not shown).

Retrovirus-mediated expression of dominant-negative BMP receptors causes phenotypic alterations similar to those triggered by noggin

Although the developmental abnormalities resulting from noggin overexpression strongly suggest that the BMPs play critical regulatory roles in patterning the ventral optic cup, the evidence is nonetheless indirect in nature. We therefore sought to corroborate those findings by retrovirus-mediated overexpression of a dominant-negative construct for BMP receptor 3 (DN BRK3). RCAS DN BRK3 or the control RCAS alkaline phosphatase were injected intravitreally on E3 (HH stage 15-18), and the embryos were fixed on E6 or E8 and analyzed to ascertain the presence of colobomas and/or pathfinding defects, equivalent to those shown in Fig. 2H and 2M. The results were very similar to those obtained with RCAS noggin, since colobomas and optic nerve pathfinding abnormalities were observed in 62% (60/96) and 9% (2/23) of RCAS DN BRK3-infected eyes, respectively, while the frequencies of similar abnormalities in RCAS-noggin-infected eyes were 79% (89/113) for colobomas and 9% (8/89) for optic nerve abnormalities. The results therefore add support to the contention that BMP signaling is necessary for normal patterning and differentiation of the ventral optic cup.

DISCUSSION

We have investigated the involvement of members of the BMP family of growth factors in the development of ventral retina and related eye tissues, by ectopic overexpression of the BMP-binding protein noggin. Noggin binds with high affinity to BMP2 and BMP4, and with lower affinity to BMP7, thus preventing their binding to their cognate receptors (reviewed by Mehler et al., 1997). Noggin overexpression at early optic vesicle stages resulted in fairly widespread abnormalities in eye development, including microphthalmia and severe disruption of the developing retina, RPE and lens. Somewhat later, at optic cup stages, defects were largely restricted to the ventral retina and adjacent tissues. These abnormalities included lack of choroid fissure closure (coloboma), absence of the pecten, replacement of ventral pigmented epithelium by secondary neuroepithelium-like tissue, ventral displacement of the optic nerve head accompanied by hypoplasia or absence of the optic nerve, and intravitreal growth of an optic nerve-like structure in some of the embryos. Cell type-specific markers normally found in the ventral region of the retina and RPE were generally undetectable in the corresponding regions of noggin-treated eyes, and appeared to be replaced by ectopically expressed optic stalk markers.

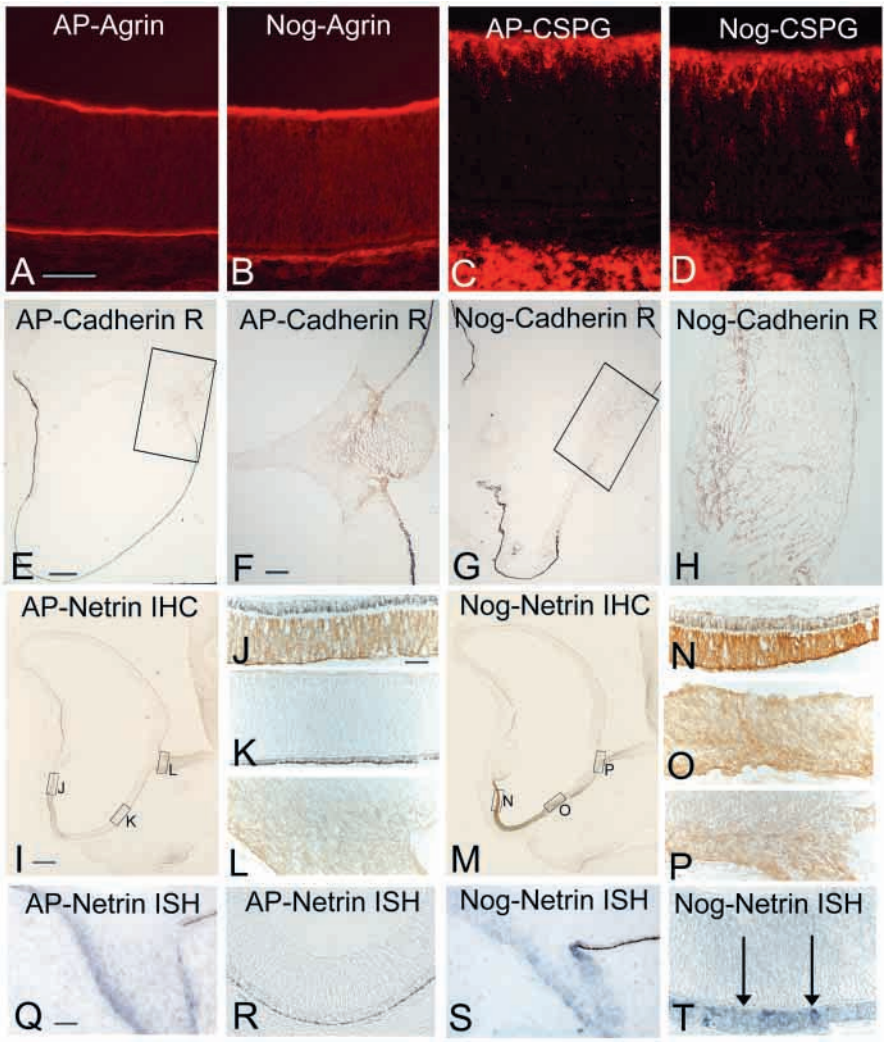

Fig. 6. Expression of axonal guidance molecules. Embryos infected at E3 were analyzed at either E6 (A,B,I-T) or E8 (C-H). (A-D) Expression of agrin (A,B) and chondroitin sulfate proteoglycan (C,D). No differences were observed between alkaline phosphatase- (A,C) and noggin- (B,D) infected embryos. (E-H) R-cadherin immunoreactivity. R-cadherin appeared localized to the pecten, optic nerve head and adjacent RPE in alkaline phosphatase-infected embryos (E,F), but was present in a much wider and more diffuse region in noggin-infected eyes (G,H). (I-P) Netrin immunoreactivity. Signals were localized to the optic nerve and ventral region of the presumptive ciliary body of alkaline phosphatase controls (I-L), but encompassed the entire ventral retina and secondary neuroepithelium of noggin-infected eyes (M-P). (Q-T) *Netrin 1* mRNA expression. *Netrin 1*-positive cells were localized to the optic stalk in both alkaline phosphatase- (Q) and noggin- (S) infected embryos, but the latter also showed positive cells in the secondary neuroepithelium (arrows, T). AP, alkaline phosphatase infected; Nog, noggin infected; CSPG, chondroitan sulfate proteoglycan; IHC, immunohistochemistry; ISH, in situ hybridization. Scale bars: A-D, 50 μm; F,H, 100 μm; E,G, 500 μm; I,M, 500 μm; J-L, N-P, 50 μm; Q-T, 50 μm.

Signaling molecules involved in the regulation of ventral retina development

Retinoic acid and sonic hedgehog have been implicated in the regulation of ventral retinal development (Hyatt et al., 1992; Hyatt et al., 1996; Kastner et al., 1994; Marsh-Armstrong et al., 1994; Zhang and Yang, 2001), but BMPs in general, and BMP4 in particular, have hitherto been described predominantly as regulators of dorsal retinal development (Koshiba-Takeuchi et al., 2000; Zhang and Yang, 2001). The capacity of the BMPs to inhibit the ventralization of the retina through the regulation of the transcription factor TBX5 has been recognized (Koshiba-Takeuchi et al., 2000), but it has been proposed that these potential effects are normally kept in check by the recently discovered BMP antagonist ventroptin (Sakuta et al., 2001), and/or by SHH (Zhang and Yang, 2001). Endogenous BMPs could conceivably also be inhibited by several other BMP binding proteins expressed in various regions of the developing optic cup, including follistatin, follistatin-like protein (flik), DAN, chordin and noggin itself (Belecky-Adams et al., 1999; Belecky-Adams and Adler, 2001; Eimon and Harland, 2001; Ogita et al., 2001; Sakuta et al., 2001). Nevertheless, exogenous noggin does have very dramatic and specific effects on the differentiation of various ventral eye structures, including retina, RPE, optic nerve and pecten. This is consistent with the finding that, at early stages of development, BMP receptors IA and IB are predominantly if not exclusively localized to the ventral retina and optic stalk (Belecky-Adams and Adler, 2001). Together with the finding that specific blockage of BMP signaling through the use of a dominant negative receptor had phenotypic consequences equivalent to those of noggin overexpression, these data suggest that endogenous BMPs do have direct effects on the development of the ventral retina (besides and beyond retinal specification along the dorsoventral axis), and do so despite the above mentioned effects of ventroptin and SHH.

The notion that BMPs may have direct effects on the ventral retina may be considered surprising, given that they are usually associated with dorsal retinal development (Koshiba-Takeuchi et al., 2000; Sakuta et al., 2001; Zhang and Yang, 2001). Studies of dorsal retina development, however, have largely focused on BMP4 (Koshiba-Takeuchi et al., 2000; Sakuta et al., 2001; Zhang and Yang, 2001), but it must be noted that several other BMPs are found in the retina and neighboring tissues (Belecky Adams and Adler, 2001). Particularly noteworthy among them is BMP7, which at early stages of development is expressed near the optic stalk and in the ventral pigmented epithelium (Belecky-Adams and Adler, 2001; Vogel-Hopker, 2000). It must be reiterated here, moreover, that

BMP receptors are expressed predominantly (receptor IA) or exclusively (receptor IB) in the ventral retina and optic stalk at those same developmental stages. The involvement of BMPs in the development of both dorsal and ventral structures of a neural organ would not be without precedent, moreover, since a similar pattern of effects has been observed in other regions of the neural tube (Liem et al., 2000). Nevertheless, it is not inconceivable that some of the phenotypic changes observed in the ventral retina in response to noggin may be mediated by primary effects on other tissues, for example, the embryonic lens, since BMP4 and BMP7 have both been shown to play a role in lens induction and differentiation (Luo et al., 1995; Solursh et al., 1996; Karsenty et al., 1996; Jena et al., 1997; Furuta and Hogan, 1998; Wawersik et al., 1999).

SHH and the BMPs appear to have overlapping but antagonistic roles in the retina, since fairly similar ventral eye phenotypes are triggered by overexpressing SHH (Nasrallah and Golden, 2001; Zhang and Yang, 2001), and by blocking BMP signaling with noggin (present work). Precedent for such antagonism can be found in the establishment of dorsoventral polarity in the spinal cord (Liem et al., 1995; Liem et al., 1997; Liem et al., 2000; Ericson et al., 1996; Ericson et al., 1997). It is noteworthy that SHH downregulates BMP4 expression in the dorsal retina (Zhang and Yang, 2001), but we found no effects of noggin overexpression on patterns of expression of SHH or its receptor patched (which serves as an indicator of levels of SHH signaling in a tissue) (Marigo and Tabin, 1996; Goodrich et al., 1996). This would appear to suggest that exogenous SHH may act upstream of BMP4 under experimental conditions (see Zhang and Yang, 2001), but it is unclear whether similar effects occur under physiological conditions. However, SHH and BMP could interact as regulators of the transcription factors PAX2 and VAX, which are involved in ventral retina development (Torres et al., 1996; Favor et al., 1996; Otteson et al., 1998; Barbieri et al., 1999; Bertuzzi et al., 1999; Hallonet et al., 1999; Schulte et al., 1999), and have been shown to be regulated by both SHH and BMP2/4 in chick tissues (Golden et al., 1999; Groves and Bronner Fraser, 2000; Zhang et al., 2000), and zebrafish (Ekker et al., 1995; MacDonald et al., 1995; Ungar and Moon, 1996). It has also been suggested that retinoic acid may act in parallel to SHH and BMP, at least in the neural tube (Pierani et al., 1999). This could conceivably apply to the retina as well, since retinoic acid acts as a ventralizing factor (Hyatt et al., 1992; Kastner et al., 1994; Marsh-Armstrong et al., 1994), albeit without mimicking exactly the effects of SHH or BMP4. These effects of RA have not been studied in the chick retina, but our observation that noggin overexpression alters the expression of the retinoic acid synthesizing enzymes ALDH1 and 6 suggests that, if RA is also involved in ventral retina development, it could act downstream from the BMPs.

The complexity of signaling systems involved in the regulation of the development of ventral ocular tissue may go beyond these apparent interactions between SHH, the BMPs, and possibly RA. Abnormalities in optic nerve formation seen upon noggin overexpression, for example, are likely influenced and/or mediated by the abnormal expression domains of netrin and/or R-cadherin, which expand from optic disc and optic stalk into the retina proper in noggin-treated eyes. Netrin plays a well characterized role in attracting ganglion cell axons into the optic nerve (Deiner et al., 1997; de la Torre et al., 1997; Livesey and Hunt, 1997; Petrausch et al., 2000; Sugimoto et al., 2001), and it is therefore conceivable that axons would become disoriented when it is produced not only in the optic stalk, but also in the ventral optic cup. The role of R-cadherin in ganglion cell axonal guidance is less clear, but it has been suggested that R-cadherin-positive glial cells are involved in the organization of the optic nerve (Gerhardt et al., 2000).

Plasticity of the ventral optic cup

Our data, together with the results of Zhang and Yang (Zhang and Yang, 2001), indicate that ventral optic cup structures retain a considerable degree of developmental plasticity up to at least E3 (HH stages 15-18), as illustrated by the changes in cell fate determined by noggin-dependent down-regulation of BMP signaling, and/or by administration of exogenous SHH. The optic stalk/optic nerve head domain expands with both treatments, for example, with concomitant aberrant growth of optic nerve. The putative ventral retina fails to develop as such in both cases, acquiring optic stalk-like properties in noggin-treated eyes, and pigment epithelium-like features after SHH treatment. The ventral RPE, in contrast, is expanded and/or hypertrophic in SHH-treated eyes, but is replaced by optic stalk-like tissue in noggin-treated embryos, and by an ectopic secondary retina in SHH loss-of-function experiments, in which some retinal markers were also observed in the putative optic stalk (Zang and Yang, 2001). Other examples of ventral optic cup plasticity resulting from RA treatment in other species have been reported (Marsh-Armstrong et al., 1994; Hyatt et al., 1996). Several of the treatments (including those reported in this paper) were accompanied by changes in the expression of topographic markers in the dorsal retina, but the latter appears to be less susceptible than the ventral retina to changes in cell fate triggered by microenvironmental signals (see Zhang and Yang, 2001).

Mechanism of coloboma formation

Although the mechanisms underlying coloboma formation are still poorly understood, significant progress has been made in the identification of transcriptional regulators and extracellular signaling molecules that can cause these serious abnormalities in eye development. Conspicuous examples of transcriptional regulator involvement are mutations/deletions in PAX2 (reviewed by Dressler and Woolf, 1999), VAX (Hallonet et al., 1999), PITX2 (Gage et al., 1999), and SOX10 (Bondurand et al., 1999), whereas signaling molecules include overexpression of SHH (Zhang and Yang, 2001), and absence of, or decreases in the availability of retinoic acid (Stull and Wikler, 2000) or the BMPs (present study). It appears likely that signaling molecules and at least some of the above-mentioned transcription factors may act in concert, because both PAX2 and VAX can be regulated by SHH, RA and BMP in the eye (Hyatt et al., 1996; Koshiba-Takeuchi et al., 2000; Zhang and Yang, 2001) (and this study). The mechanisms by which these proteins interfere with the closure of the choroid fissure are still unclear. This is still a poorly understood phenomenon that, like similar morphogenetic events, may involve complex changes in cell adhesion, cell shape, cell proliferation, cell death, and/or extracellular matrix molecules. It has been proposed that degradation of the laminin-containing basement membrane in the area of apposition between the two opposing faces of the retina and RPE is necessary for this fusion to occur (Torres et

al., 1996), and we have in fact observed the persistence of the laminin membrane in noggin-treated eyes. However, it remains to be determined whether this is causally related to the genesis of the coloboma, either by itself or in combination with other mechanisms.

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REFERENCES

Austin, C. P., Feldman, D. E., Ida, J. A. and Cepko, C. L. (1995). Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. *Development* **121**, 3637-3650.

Barbieri, A. M., Lupo, G., Bulfone, A., Andreazzoli, M., Mariani, M., Fougerousse, F., Consalez, G. G., Borsani, G., Beckmann, J. S., Barsacchi, G. et al. (1999). A homeobox gene, VAX2, controls the patterning of the eye dorsoventral axis. *Proc. Natl. Acad. Sci. USA* **96**, 10729-10734.

Belecky-Adams, T. and Adler, R. (2001). Developmental expression patterns of bone morphogenetic proteins, receptors, and binding proteins in the chick retina. *J. Comp. Neurol.* **430**, 562-572.

Belecky-Adams, T., Cook, B. and Adler, R. (1996). Correlations between terminal mitosis and differentiated fate of retinal precursor cells in vivo and in vitro: analysis with the "window-labeling" technique. *Dev. Biol.* **178**, 304-315.

Belecky-Adams, T. L., Scheurer, D. and Adler, R. (1999). Activin family members in the developing chick retina: Expression patterns, protein distribution, and in vitro effects. *Dev. Biol.* **210**, 107-123.

Belecky-Adams, T., Tomarev, S., Li, H. S., Ploder, L., McInnes, R. R., Sundin, O. and Adler, R. (1997). PAX6, Prox 1, and Chx10 homeobox gene expression correlates with phenotypic fate of retinal precursor cells. *Invest. Ophthal. Vis. Sci.* **38**, 1293-1303.

Bertuzzi, S., Hindges, R., Mui, S. H., O'Leary, D. D. and Lemke, G. (1999). The homeodomain protein VAX1 is required for axon guidance and major tract formation in the developing forebrain. *Genes Dev.* **13**, 3092-3105.

Bondurand, N., Kuhlbrodt, K., Pingault, V., Enderich, J., Sajus, M., Tommerup, N., Warburg, M., Hennekam, R. C., Read, A. P., Wegner, M. et al. (1999). A molecular analysis of the yemenite deaf-blind hypopigmentation syndrome: SOX10 dysfunction causes different neurocristopathies. *Hum. Mol. Genet.* **8**, 1785-1789.

Deiner, M. S., Kennedy, T. E., Fazeli, A., Serafini, T., Tessier-Lavigne, M. and Sretavan, D. W. (1997). Netrin-1 and DCC mediate axon guidance locally at the optic disc: loss of function leads to optic nerve hypoplasia. *Neuron* **19**, 575-589.

de la Torre, J. R., Hopker, V. H., Ming, G. L., Poo, M. M., Tessier-Lavigne, M., Hemmati-Brivanlou, A. and Holt, C. E. (1997). Turning of retinal growth cones in a netrin-1 gradient mediated by the netrin receptor DCC. *Neuron* **19**, 1211-1224.

Dressler, G. R. and Woolf, A. S. (1999). Pax2 in development and renal disease. *Int. J. Dev. Biol.* **43**, 463-468.

Eimon, P. M. and Harland, R. M. (2001). Xenopus Dan, a member of the Dan gene family of BMP antagonists, is expressed in derivatives of the cranial and trunk neural crest. *Mech. Dev.* **107**, 187-189.

Ekker, S. C., Ungar, A. R., Greenstein, P., von Kessler, D. P., Porter, J. A., Moon, R. T. and Beachy, P. A. (1995). Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. *Curr. Biol.* **5**, 944-955.

Ericson, J., Morton, S., Kawakami, A., Roelink, H. and Jessell, T. M. (1996). Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. *Cell* **87**, 661-673.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded SHH signaling. *Cell* **90**, 169-180.

Favor, J., Sandulache, R., Neuhauser-Klaus, A., Pretsch, W., Chatterjee, B., Senft, E., Wurst, W., Blanquet, V., Grimes, P., Sporle, R. et al. (1996). The mouse Pax2(1Neu) mutation is identical to a human PAX2 mutation in a family with renal-coloboma syndrome and results in developmental defects of the brain, ear, eye, and kidney. *Proc. Natl. Acad. Sci. USA* **93**, 13870-13875.

Furuta, Y. and Hogan, B. L. (1998). BMP4 is essential for lens induction in the mouse embryo. *Genes Dev.* **12**, 3764-3775.

Gage, P. J., Suh, H. and Camper, S. A. (1999). Dosage requirement of Pitx2 for development of multiple organs. *Development* **126**, 4643-4651.

Gerhardt, H., Rascher, G., Schuck, J., Weigold, U., Redies, C. and Wolburg, H. (2000). R- and B-cadherin expression defines subpopulations of glial cells involved in axonal guidance in the optic nerve head of the chicken. *GLIA* **31**, 131-143.

Golden, J. A., Bracilovic, A., McFadden, K. A., Beesley, J. S., Rubenstein, J. L. and Grinspan, J. B. (1999). Ectopic bone morphogenetic proteins 5 and 4 in the chicken forebrain lead to cyclopia and holoprosencephaly. *Proc. Natl. Acad. Sci. USA* **96**, 2439-2444.

Goodhill, G. J. and Richards, L. J. (1999). Retinotectal maps: molecules, models and misplaced data. *Trends Neurosci.* **22**, 529-534.

Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. A. and Scott, M. P. (1996). Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. *Genes Dev.* **10**, 301-312.

Groves, A. K. and Bronner-Fraser, M. (2000). Competence, specification and commitment in otic placode induction. *Development* **127**, 3489-3499.

Grun, F., Hirose, Y., Kawauchi, S., Ogura, T. and Umesono, K. (2000). Aldehyde dehydrogenase 6, a cytosolic retinaldehyde dehydrogenase prominently expressed in sensory neuroepithelia during development. *J. Biol. Chem.* **275**, 41210-41218.

Hallonet, M., Hollemann, T., Pieler, T. and Gruss, P. (1999). VAX1, a novel homeobox-containing gene, directs development of the basal forebrain and visual system. *Genes Dev.* **13**, 3106-3114.

Hero, I. (1989). The optic fissure in the normal and microphthalmic mouse. *Exp. Eye Res.* **49**, 229-239.

Hero, I. (1990). Optic fissure closure in the normal cinnamon mouse. An ultrastructural study. *Invest. Ophthalmol. Vis. Sci.* **31**, 197-216.

Hero, I., Farjah, M. and Scholtz, C. L. (1991). The prenatal development of the optic fissure in colobomatous microphthalmia. *Invest. Ophthalmol. Vis. Sci.* **32**, 2622-2635.

Hughes, S. H., Greenhouse, J. J., Petropoulos, C. J. and Sutrave, P. (1987). Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. *J. Virol.* **61**, 3004-3012.

Hyatt, G. A., Schmitt, E. A., Marsh-Armstrong, N., McCaffery, P., Drager, U. C. and Dowling, J. E. (1996). Retinoic acid establishes ventral retinal characteristics. *Development* **122**, 195-204.

Hyatt, G. A., Schmitt, E. A., Marsh-Armstrong, N. R. and Dowling, J. E. (1992). Retinoic acid-induced duplication of the zebrafish retina. *Proc. Natl. Acad. Sci. USA* **89**, 8293-8297.

Jena, N., Martin-Seisdedos, C., McCue, P., Corce, C. M. (1997). BMP7 null mutation in mice: developmental defects in skeleton, kidney, and eye. *Exp. Cell Res.* **230**, 28-37.

Karsenty, G., Luo, G., Hofmann, C. and Bradley, A. (1996). BMP 7 is required for nephrogenesis, eye development, and skeletal patterning. *Ann. NY Acad. Sci.* **785**, 98-107.

Kastner, P., Grondona, J. M., Mark, M., Gansmuller, A., LeMeur, M., Decimo, D., Vonesch, J. L., Dolle, P. and Chambon, P. (1994). Genetic analysis of RXR alpha developmental function: convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. *Cell* **78,** 987-1003.

Koshiba-Takeuchi, K., Takeuchi, J. K., Matsumoto, K., Momose, T., Uno, K., Hoepker, V., Ogura, K., Takahashi, N., Nakamura, H., Yasuda, K. et al. (2000). Tbx5 and the retinotectum projection. *Science* **287**, 134-137.

Li, H. S., Yang, J. M., Jacobson, R. D., Pasko, D. and Sundin, O. (1994). PAX6 is first expressed in a region of ectoderm anterior to the early neural plate: implications for stepwise determination of the lens. *Dev. Biol.* **162**, 181-194.

Liem, K. F., Jr, Jessell, T. M. and Briscoe, J. (2000). Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites. *Development* **127**, 4855-4866.

Liem, K. F., Tremml, G. and Jessell, T. M. (1997). A role for the roof plate and its resident TGFbeta-related proteins in neuronal patterning in the dorsal spinal cord. *Cell* **91**, 127-138.

Liem, K. F., Tremml, G., Roelink, H. and Jessell, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82**, 969-979.

Livesey, F. J. and Hunt, S. P. (1997). Netrin and netrin receptor expression in the embryonic mammalian nervous system suggests roles in retinal, striatal, nigral, and cerebellar development. *Mol. Cell Neurosci.* **8**, 417-429.

Luo, G., Hofmann, C., Bronckers, A. L., Sohocki, M., Bradley, A., Karsenty, G. (1995). BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. *Genes Dev.* **9**, 2808-2820.

Macdonald, R., Barth, K. A., Xu, Q., Holder, N., Mikkola, I. and Wilson, S. W. (1995). Midline signalling is required for Pax gene regulation and patterning of the eyes. *Development* **121**, 3267-3278.

Manitt, C., Colicos, M. A., Thompson, K. M., Rousselle, E., Peterson, A. C. and Kennedy, T. E. (2001). Widespread expression of netrin-1 by neurons and oligodendrocytes in the adult mammalian spinal cord. *J. Neurosci*. **21**, 3911-3922.

Marigo, V. and Tabin, C. J. (1996). Regulation of patched by sonic hedgehog in the developing neural tube. *Proc. Natl. Acad. Sci. USA* **93**, 9346-9351.

Marsh-Armstrong, N., McCaffery, P., Gilbert, W., Dowling, J. E. and Drager, U. C. (1994). Retinoic acid is necessary for development of the ventral retina in zebrafish. *Proc. Natl. Acad. Sci. USA* **91**, 7286-7290.

McCaffery, P. and Drager, U. C. (1993). Retinoic acid synthesis in the developing retina. *Adv. Exp. Med. Biol.* **328**, 181-190.

McCaffery, P., Lee, M. O., Wagner, M. A., Sladek, N. E. and Drager, U. C. (1992). Asymmetrical retinoic acid synthesis in the dorsoventral axis of the retina. *Development* **115**, 371-382.

McCaffery, P., Tempst, P., Lara, G. and Drager, U. C. (1991). Aldehyde dehydrogenase is a positional marker in the retina. *Development* **112**, 693-702.

McCaffery, P., Wagner, E., O'Neil, J., Petkovich, M. and Drager, U. C. (1999). Dorsal and ventral retinal territories defined by retinoic acid synthesis, break-down and nuclear receptor expression. *Mech. Dev.* **82**, 119-130.

Mehler, M. F., Mabie, P. C., Zhang, D. and Kessler, J. A. (1997). Bone morphogenetic proteins in the nervous system. *Trends Neurosci.* **20**, 309-317.

Mey, J., McCaffery, P. and Drager, U. C. (1997). Retinoic acid synthesis in the developing chick retina. *J. Neurosci.* **17**, 7441-7449.

Mey, J., McCaffery, P. and Klemeit, M. (2001). Sources and sink of retinoic acid in the embryonic chick retina: distribution of aldehyde dehydrogenase activities, CRABP-I, and sites of retinoic acid inactivation. *Brain Res. Dev. Brain Res.* **127**, 135-148.

Mic, F. A., Molotkov, A., Fan, X., Cuenca, A. E. and Duester, G. (2000). RALDH3, a retinaldehyde dehydrogenase that generates retinoic acid, is expressed in the ventral retina, otic vesicle and olfactory pit during mouse development. *Mech. Dev.* **97**, 227-230.

Mochii, M., Takeuchi, T., Kodama, R., Agata, K. and Eguchi, G. (1988). The expression of melanosomal matrix protein in the transdifferentiation of pigmented epithelial cells into lens cells. *Cell Diff.* **23**, 133-141.

Morgan, B. A. and Fekete, D. M. (1996). Manipulating gene expression with replication-competent retroviruses. *Methods Cell Biol*. **51**, 185-218.

Nasrallah, I. and Golden, J. A. (2001). Brain, eye, and face defects as a result of ectopic localization of Sonic hedgehog protein in the developing rostral neural tube. *Teratology* **64**, 107-113.

Nornes, H. O., Dressler, G. R., Knapik, E. W., Deutsch, U. and Gruss, P. (1990). Spatially and temporally restricted expression of Pax2 during murine neurogenesis. *Development* **109**, 797-809.

Ogita, J., Isogai, E., Sudo, H., Sakiyama, S., Nakagawara, A. and Koseki, H. (2001). Expression of the Dan gene during chicken embryonic development. *Mech. Dev.* **109**, 263-265.

Ohsaki, K., Morimitsu, T., Ishida, Y., Kominami, R. and Takahashi, N. (1999). Expression of the VAX family homeobox genes suggests multiple roles in eye development. *Genes Cells* **4**, 267-276.

O'Leary, D. D. and Wilkinson, D. G. (1999). Eph receptors and ephrins in neural development. *Curr. Opin. Neurobiol.* **9**, 65-73.

Otteson, D. C., Shelden, E., Jones, J. M., Kameoka, J. and Hitchcock, P. F. (1998). Pax2 expression and retinal morphogenesis in the normal and Krd mouse. *Dev. Biol.* **193**, 209-224.

Petrausch, B., Jung, M., Leppert, C. A. and Stuermer, C. A. (2000). Lesion-induced regulation of netrin receptors and modification of netrin-1 expression in the retina of fish and grafted rats. *Mol. Cell Neurosci.* **16**, 350-364.

Pierani, A., Brenner-Morton, S., Chiang, C. and Jessell, T. M. (1999). A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* **97**, 903-915.

Redies, C. and Takeichi, M. (1993). N- and R-cadherin expression in the optic nerve of the chicken embryo. *GLIA* **8**, 161-171.

Sakuta, H., Suzuki, R., Takahashi, H., Kato, A., Shintani, T., Iemura, S., Yamamoto, T. S., Ueno, N. and Noda, M. (2001). Ventroptin: a BMP-4 antagonist expressed in a double-gradient pattern in the retina. *Science* **293**, 111-115.

Schulte, D., Furukawa, T., Peters, M. A., Kozak, C. A. and Cepko, C. L. (1999). Misexpression of the Emx-related homeobox genes cVAX and mVAX2 ventralizes the retina and perturbs the retinotectal map. *Neuron* **24**, 541-553.

Schwarz, M., Cecconi, F., Bernier, G., Andrejewski, N., Kammandel, B., Wagner, M. and Gruss, P. (2000). Spatial specification of mammalian eye territories by reciprocal transcriptional repression of Pax2 and Pax6. *Development* **127**, 4325-4334.

Selleck, M. A. (1996). Culture and microsurgical manipulation of the early avian embryo. *Methods Cell Biol*. **51**, 1-21.

Smith, W. C. (1999). TGF beta inhibitors. New and unexpected requirements in vertebrate development. *Trends Genet.* **15**, 3-5.

Snow, R. L. and Robson, J. A. (1994). Ganglion cell neurogenesis, migration and early differentiation in the chick retina. *Neuroscience* **58**, 399-409.

Solursh, M., Langille, R. M., Wood, J. and Sampath, T. K. (1996). Osteogenic protein-1 is required for mammalian eye development. *Biochem. Biophys. Res. Commun.* **218**, 438-443.

Stull, D. L. and Wikler, K. C. (2000). Retinoid-dependent gene expression regulates early morphological events in the development of the murine retina. *J. Comp. Neurol.* **417**, 289-298.

Sugimoto, Y., Taniguchi, M., Yagi, T., Akagi, Y., Nojyo, Y. and Tamamaki, N. (2001). Guidance of glial precursor cell migration by secreted cues in the developing optic nerve. *Development* **128**, 3321-3330.

Suzuki, R., Shintani, T., Sakuta, H., Kato, A., Ohkawara, T., Osumi, N. and Noda, M. (2000). Identification of RALDH-3, a novel retinaldehyde dehydrogenase, expressed in the ventral region of the retina. *Mech. Dev.* **98**, 37-50.

Torres, M., Gomez-Pardo, E. and Gruss, P. (1996). Pax2 contributes to inner ear patterning and optic nerve trajectory. *Development* **122**, 3381-3391.

Ungar, A. R. and Moon, R. T. (1996). Inhibition of protein kinase A phenocopies ectopic expression of hedgehog in the CNS of wild-type and cyclops mutant embryos. *Dev. Biol.* **178**, 186-191.

Vogel-Hopker, A., Momose, T., Rohrer, H., Yasuda, K., Ishihara, L. and Rapaport, D. H. (2000). Multiple functions of fibroblast growth factor-8 (FGF8) in chick eye development. *Mech. Dev.* **94**, 25-36.

Waid, D. K. and McLoon, S. C. (1995). Immediate differentiation of ganglion cells following mitosis in the developing retina. *Neuron* **14**, 117-124.

Wawersik, S., Purcell, P., Rauchman, M., Dudley, A. T., Robertson, E. J. and Maas, R. (1999). BMP7 acts in murine lens placode development. *Dev. Biol.* **207**, 176-188.

Wohrn, J. C., Puelles, L., Nakagawa, S., Takeichi, M. and Redies, C. (1998). Cadherin expression in the retina and retinofugal pathways of the chicken embryo. *J. Comp. Neurol.* **396**, 20-38.

Wolburg, H., Liebner, S., Reichenbach, A. and Gerhardt, H. (1999). The pecten oculi of the chicken: a model system for vascular differentiation and barrier maturation. *Int. Rev. Cytol.* **187**, 111-159.

Zhang, X. M., Lin, E. and Yang, X. J. (2000). Sonic hedgehog-mediated ventralization disrupts formation of the midbrain-hindbrain junction in the chick embryo. *Dev. Neurosci.* **22**, 207-216.

Zhang, X. M. and Yang, X. J. (2001). Temporal and spatial effects of Sonic hedgehog signaling in chick eye morphogenesis. *Dev. Biol.* **233**, 271-290.