

# Microphthalmia Resulting from *Msx2*-Induced Apoptosis in the Optic Vesicle

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**PURPOSE.** Microphthalmia is a relatively common ocular malformation. Molecular mechanisms that lead to this dire condition are largely unknown. *Msx* genes have been shown to be expressed in the developing eye. In the *Msx1;Msx2*, double mutant mouse, eye development arrests early in embryogenesis. To investigate possible functions of *Msx2* in early ocular development, we created transgenic animals that overexpress *Msx2*.

**METHODS.** *Msx2* transgenic embryos and nontransgenic littermates were examined histopathologically. The effect of *Msx2* overexpression on retinal cell proliferation was assayed by bromodeoxyuridine (BrdU) incorporation and immunohistochemical staining. Apoptosis was determined by TUNEL labeling. Expression of retina and retinal pigmented epithelium (RPE)-specific genes was investigated by performing in situ hybridization or immunohistochemical staining.

**RESULTS.** Forced expression of the *Msx2* gene resulted in optic nerve aplasia and microphthalmia in all transgenic animals. In developing retinas of *Msx2* transgenic animals, proliferation was significantly reduced and increased numbers of retinal cells underwent apoptosis. Marker analysis showed suppression of *Bmp4* and induction of *Bmp7* gene expression in the optic vesicle. Ectopic concurrent expression of the RPE cell markers *Cx43* and *Trp-2* in the neural retinal layer suggests cell fate respecification.

**CONCLUSION.** These results indicate that forced expression of *Msx2* perturbs BMP signaling in the developing eye and is accompanied by an increase in retinal cell death and a reduction in cell proliferation. Thus, deregulated *Msx2* gene expression may be a plausible genetic mechanism by which the autosomal dominant form of congenital microphthalmia may

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The morphogenesis of the eye is dictated by a series of reciprocal tissue–tissue interactions. These interactions begin when the bulging diencephalic neural ectoderm induces the overlying surface ectoderm to become the lens placode. When the anterior neural tube becomes completely sealed, the lens ectoderm and the contacting neural ectoderm invaginate to form a three-layered optic cup. The epidermal component develops into the lens, the inner layer of the neural ectoderm later differentiates into the highly structured and multilayered neural retina, and the outer layer forms the retinal pigmented epithelium.<sup>1–3</sup> Although the morphologic development of the vertebrate eye has been well described, the molecular basis underlying ocular development and malformations has just begun to be understood.

Recent studies have demonstrated that early ocular developmental processes are controlled by a complex network of transcriptional factors, cell cycle regulators, and diffusible signaling molecules.<sup>3,4</sup> Among these regulatory molecules are *Pax6*,<sup>5,6</sup> *Pax2*,<sup>7</sup> *Six3* and *Six6*,<sup>8,9</sup> *Vax1*,<sup>10</sup> *Mitf*,<sup>11–13</sup> *Chx10*,<sup>14,15</sup> *Lhx2*,<sup>16</sup> *Rx*,<sup>17</sup> *Otx1*,<sup>18</sup> *Otx2*,<sup>19–22</sup> *cyclin D1*,<sup>23,24</sup> *p27<sup>kip1</sup>*,<sup>25–27</sup> *BMP4*,<sup>28–30</sup> and *BMP7*.<sup>31–36</sup> Together, these molecules craft different ocular compartments, regulate cell proliferation and apoptosis, and specify retinal cell identities.

*Msx2*, one of three related mammalian genes—*Msx1*, *Msx2*, and *Msx3*—that constitute the *msb* gene family<sup>37</sup> has been shown to be expressed, though at low levels, in the developing optic vesicle.<sup>38,39</sup> *Msx1;Msx2* double-null mutants showed arrest in eye development.<sup>40</sup> Together, these observations lead to the suggestion that *Msx* genes are functionally involved in the development of the vertebrate eye.

*Msx2* encodes a homeodomain transcription factor that is known to play a critical role in regulating calvarial bone and suture development, mammary gland genesis, and hair follicle formation.<sup>41–47</sup> Null mutations in the *Msx2* gene have been shown to arrest hair follicle and mammary gland development in the mouse and to delay and prevent ossification of the parietal foramina in both mouse and man.<sup>46,47</sup> Furthermore, overexpression of the *Msx2* gene in transgenic animals resulted in hypokeratosis of the skin and enhancement of ossification of skull bones as a result of enhanced proliferation of osteoprogenitors.<sup>42,43,44</sup> A hypermorphic mutation in *MSX2* is the cause of Boston type craniosynostosis.<sup>41</sup> These phenotypes were consistent with the expression profile of the *Msx2* gene during development and its role in regulating cell proliferation and differentiation processes.

Programmed cell death (PCD), or apoptosis, has been shown to occur during normal ocular development.<sup>48,49</sup> Scattered apoptotic cells have been detected in the mouse optic vesicle and the optic stalk as early as embryonic day (E)9. This is followed by spatially distributed apoptotic events in specific regions of the developing retina, lens, and optic stalk.<sup>49</sup> In eye development, apoptosis is thought to control cell number and ocular shape by eliminating excessive and abnormal cells. Most

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recent studies have focused on the role of apoptosis in the retinal ganglion cells (RGCs) and photoreceptor cells of the mature retina, because loss of these cell populations is found in several common retinal diseases.<sup>50</sup> *Msx* genes, together with *Bmp4* or *Bmp7*, have been shown to regulate cell death programs in specific tissues during development including cranial neural crest cells, neuronal precursors, and vertebrate retinal cells.<sup>29,30,51-54</sup> Ectopic expression of *Msx2* has also been shown to induce apoptosis in a number of in vivo and in vitro systems.<sup>54-56</sup>

The present study provided evidence showing that *Msx2* functions as a cell death-promoting factor in the developing murine optic vesicle. Deregulated expression of the *Msx2* gene in transgenic mice can alter *Bmp4* and *Bmp7* gene expression, initiate cellular apoptosis in the developing neural retina, and cause microphthalmia.

## METHODS

### Transgene Constructs

A *SpeI*-*NotI* fragment, which contains part of the first exon and minimal promoter of the *Msx2* gene, was released from pAB1.6 and subcloned into pBluescript SKII (Stratagene, La Jolla, CA) to generate pSK-SN. Subsequently, a 5-kb *XhoI*-*SpeI* fragment covering most of the upstream regulatory sequences for the *Msx2* gene was subcloned into the *SpeI*-*XhoI* site of pSK-SN to generate pSK-SNNX. The *NotI*-*XhoI* fragment from pSK-SNNX was then moved into *NotI*-*XhoI* sites of pGem11Zf (Promega, Madison, WI) to make pGem11ZF-NX-#3. To create the coding region for the transgene, a *Bss*HII2-*XhoI* fragment covering the entire coding region, intron, and 3' sequences of the *Msx2* gene was cloned into the *Hind*III-*XhoI* sites of pSP73 (Promega) to generate pClone17. A *Sall*-*NdeI* fragment that contains the entire first exon and intron was moved from pClone17 and replaced the *KpnI*-*NdeI* fragment of pCRIIc-*Msx2*. An *Sall* site was regenerated as a result. The intron was shortened to approximately 500 bp by removing a 3.1-kb *KpnI* fragment from this plasmid to generate pCRIIWTΔKpnI. A PCR product containing the 3' UTR and polyadenylation signal from the human *MSX2* gene was then cloned into the *EcoRI*-*XhoI* site of pCRIIWTΔKpnI to create pCRIIWTΔKpnIhpa. To complete the construction of p*Msx2*WThpa, the *Sall*-*NotI* fragment in pCRIIWTΔKpnIhpa, was replaced by the *Sall*-*NotI* DNA fragment from pGem11ZF-NX-#3. For microinjection, the transgene was freed from vector sequences by cleaving the plasmid with *XhoI*.

### Generation of Transgenic Animals

Experimental animals used in this study were handled in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Fertilized oocytes were obtained from superovulated, 6-week old (C57BL/6J x CBA/J)F1 females impregnated by (C57BL/6J x CBA/J)F1 adult males (Jackson Laboratory, Bar Harbor, ME). Pseudopregnant females for embryo transfer were produced by matings between CD1 adult females and vasectomized CD1 adult males (Charles River, Wilmington, MA). Microinjection and oviduct transfer of injected zygotes was performed as described.<sup>44</sup> Usually, both procedures were performed on the same day. The concentration of DNA used for injection was 1 μg/mL. Genotyping was achieved with Southern blot hybridization.

### In Situ Hybridization

Embryos were fixed for 4 hours in 4% paraformaldehyde in phosphate-buffered saline (PBS). After fixation, samples were washed in PBS and then 0.85% NaCl, dehydrated through graded ethanol, cleared in xylene, and embedded in paraffin. Sections (7 μm) were collected on 3-amiopropyltriethoxysilane (TESPA)-treated slides (Sigma), and in situ hybridization was performed as described.<sup>57</sup> <sup>33</sup>P-labeled RNA probes

were used at 5 × 10<sup>4</sup> cpm/μL. Emulsion-coated slides were exposed for 12 to 14 days.

Probes for *TRP-2* were provided by Ian J. Jackson (Medical Research Council [MRC], Edinburgh, UK). The plasmids p5A7 and p5A3 were used to generate antisense and sense probes, respectively.<sup>58</sup> Connexin43 probe was provided by Cecilia W. Low (University of Pennsylvania, Philadelphia, PA).<sup>59</sup> Pax6 probe was obtained from Richard Maas (Harvard Medical School, Boston, MA).

Nonradioactive in situ was performed as described.<sup>60</sup> Probe for *Bmp4* was provided by Yiping Chen (Tulane University, New Orleans, LA) and probe for *Bmp7* was provided by Karen E. Lyons (University of Los Angeles, Los Angeles, CA).

### Immunohistochemistry

Embryos were fixed for 4 hours in Carnoy fixative. Samples were then washed with 70% ethanol and dehydrated through graded ethanol, cleared in xylene, and embedded in paraffin. Sections (7 μm) were collected on TESPA-treated slides. Immunostaining was performed using after antibodies: rabbit polyclonal anti-neurofilament antibody (Sigma), polyclonal rabbit anti-Pax-2 (1:100; Berkeley Antibody Co., Richmond, CA) and monoclonal anti-Pax6 (1:100; Developmental Studies Hybridoma Bank, Iowa City, IA). Antigen retrieval to detect Pax2 and Pax6 was performed by boiling sections in 0.1 M sodium citrate (pH 6.8) for 10 minutes before adding blocking reagents. After the addition of primary antibodies, sections were incubated in a humidified chamber overnight at room temperature. Preimmune serum or IgG was used as the negative control. A kit (HistoMouse; Zymed, San Francisco, CA) was used for subsequent signal amplification and color development.

### β-Galactosidase Wholemount Staining

Embryos were fixed for 10 minutes in 4% paraformaldehyde in PBS. After fixation, samples were washed in PBS and stained overnight in X-Gal staining solution as previously described.<sup>44,61</sup>

### BrdU Labeling of Proliferating Cells

Timed-pregnant mice were injected intraperitoneally with 100 μg of BrdU per gram of body weight. One hour later, animals were killed. Embryos were dissected and fixed in 4% paraformaldehyde for 2 hours. Embryos were then dehydrated through graded ethanol and embedded in paraffin. Sections were cut (7 μm), deparaffinized, and soaked in 3% hydrogen peroxide in methanol for 10 minutes. They were washed three times in PBS and treated with proteinase K (10 μg/mL in 50 mM Tris-HCl, 5 mM EDTA [pH 8.0]) for 20 minutes at 37°C. Subsequently, to depurinate DNA, sections were incubated in freshly prepared 2 N HCl for 45 minutes at room temperature and neutralized in 0.1 M sodium borate (pH 8.5) for 10 minutes. Sections were then rinsed three times in 1× PBST (1× PBS, 0.1% Tween 20). Immunodetection of BrdU was performed as just described, with mouse monoclonal anti-BrdU antibody (Zymed).

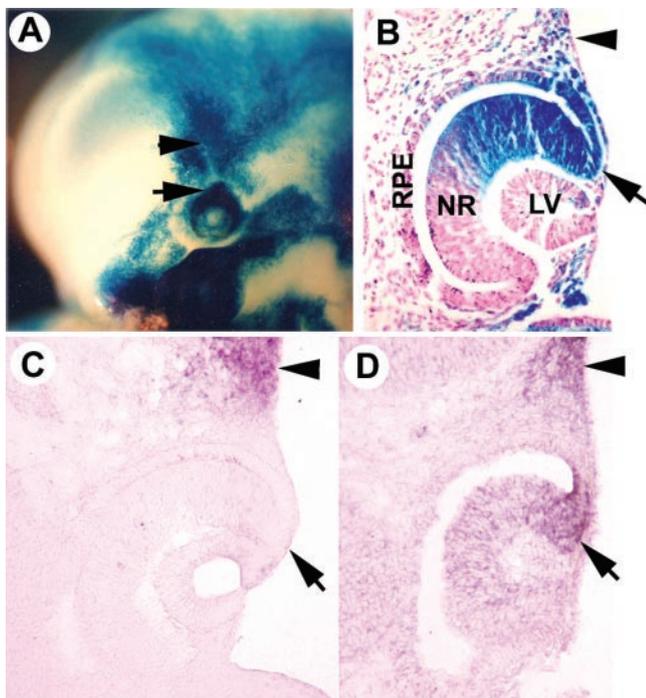
### Labeling of Apoptotic Cells

Apoptotic cells were detected by terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL), as described,<sup>62</sup> by using an apoptosis detection kit (Fluorescein In situ Cell Death Detection Kit; Roche Diagnostics, Indianapolis, IN). Briefly, tissue sections were treated with proteinase K. Fragmented DNA was labeled with fluorescein-dUTP, using terminal transferase. Labeled cells were visualized with a fluorescence microscope (Carl Zeiss Meditec, Thornwood, NY), and images were captured with a charge-coupled device camera (Spot; Diagnostic Instruments, Sterling Heights, MI).

## RESULTS

### Description of *Msx2* Transgenic Animals

Previously, we have identified a 5.2-kb promoter sequence in the *Msx2* gene that, when fused to a *lacZ* reporter and an SV40



**FIGURE 1.** Expression of the *Msx2*  $-5.2/lacZ$  transgene in the developing eye. (A) Wholemount  $\beta$ -galactosidase staining (blue) to monitor the *Msx2* promoter activity in an E10 transgenic embryo. Intense staining in and around the optic placode was evident. (B) A frontal section through the eye showed intense staining in the presumptive lens ectoderm, presumptive neural retina, and the neuroepithelium, which is destined to become RPE. (C) Nonradioactive in situ hybridization with the *Msx2* riboprobe to demonstrate weak expression of the endogenous *Msx2* gene in the developing optic vesicle of a nontransgenic embryo at E10.5. Note the strong hybridization signal in the head mesenchyme (arrowhead) in contrast to a very weak hybridization signal in the dorsal neural retina (arrow) and the lens vesicle. (D) Nonradioactive in situ hybridization on the frontal section of a *Msx2* transgenic embryo using *Msx2* probe. Strong hybridization signal was detected in the head mesenchyme (arrowhead), in the dorsal neural retina (arrow) of this transgenic optic vesicle at E10.5. RPE, retinal pigmented epithelium; NR, neural retina; LV, lens vesicle.

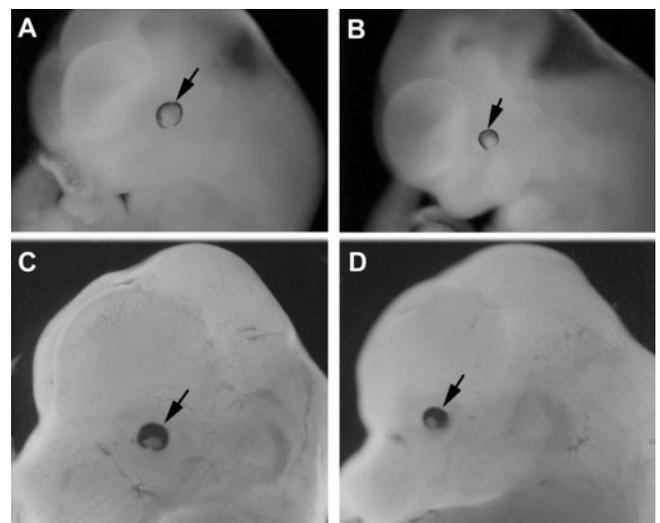
polyadenylation signal, was expressed in most neural crest-derived structures.<sup>61</sup> The  $-5.2/lacZ$  construct was also expressed in the developing eye. This 5.2-kb DNA fragment was shown to target reporter expression to the dorsal half of the neural retina in four independent transgenic lines (Fig. 1A, 1B). We used this promoter to target the *Msx2* transgene expression in the developing embryo. We generated five F0 transgenic animals. One F0 died shortly after birth and was microphthalmic. Histologic sections of the eye revealed agenesis of the retina and the lens with an intact RPE layer. One F0 appeared phenotypically normal and did not transmit the transgene to its progeny. One F0 mouse was microphthalmic and showed polydactyly. This mouse died before reaching sexual maturity. Two independent transgenic lines were established from the remaining F0 animals.

To demonstrate transgene expression, we performed RNA in situ hybridization on cryosections of transgenic and nontransgenic embryos. In contrast to a barely detectable expression level of the endogenous *Msx2* gene in the optic vesicle<sup>38,39</sup> (Fig. 1C), the level of *Msx2* transcript was increased significantly in the dorsal retina of the *Msx2* transgenic embryo (Fig. 1D). The hybridization signal in the head mesenchyme above the optic vesicle served as a positive control for relative expression (Figs. 1C, 1D). As a result of overexpression in the optic vesicle, 100% of *Msx2* transgenic animals from two sep-

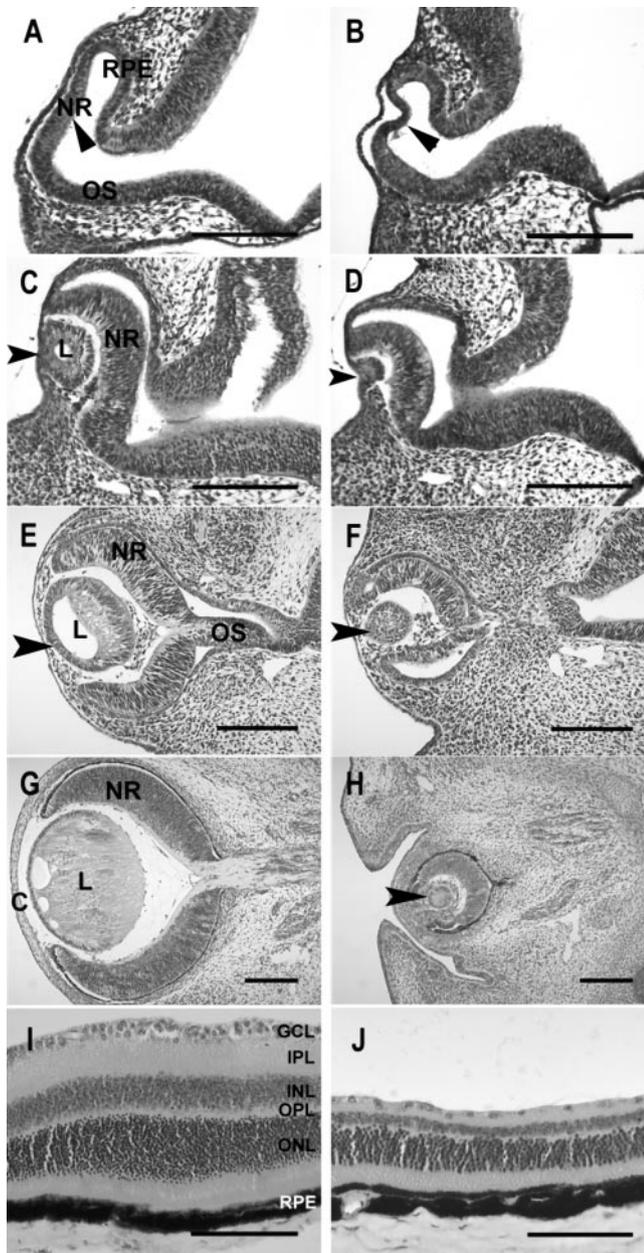
arate transgenic lines had small eyes. All transgenic animals were born with body size and weight comparable to that of nontransgenic littermates. The extent of microphthalmos was not always bilaterally symmetrical. In one transgenic line, approximately 57% of *Msx2* transgenic animals were bilaterally anophthalmic. In these mice, only the RPE was identified morphologically. In 29% of the transgenic animals, when one eye was anophthalmic, the other eye displayed a microphthalmic phenotype. The remaining 14% of transgenic animals were bilaterally microphthalmic. In the second transgenic line, approximately 95% of transgenic animals displayed bilateral microphthalmic phenotype. Our study is mostly based on the analysis of the latter transgenic line.

Optic vesicles in the *Msx2* transgenic animals appeared abnormal as early as E10 (data not shown) and assumed a hypoplastic appearance by E11. Instead of a typical square-shaped optic vesicle with a clearly defined RPE layer outlining the vesicle, as seen in the wild-type embryo (Fig. 2A), the optic vesicle of the transgenic embryo appeared smaller and oval shaped (Fig. 2B). At E14, the size of the transgenic eye was approximately one third that of the wild type and, in the most extreme case, the eye was identified only by the presence of the RPE layer and a very tiny lenslike structure (Figs. 2C, 2D and see Fig. 7A, 7B).

Histologically, abnormalities in the eyes of the transgenic embryos developed before the formation of the optic cup. At E9.5, both the presumptive retinal neural epithelium and the adjacent presumptive lens epithelium in the transgenic embryos appeared much thinner and contained fewer cells (Fig. 3B); the epithelial layers in the wild-type embryos appeared much thicker (Fig. 3A). By E10, lens induction occurred and the optic vesicle had formed although in the transgenic embryo the lens vesicle was smaller than that in the wild-type embryo (Figs. 3C, 3D). We observed optic vesicle formation in 100% of transgenic embryos ( $n = 10$ ). At E11, eye development was severely retarded (Fig. 3F). The RPE layer of the transgenic optic vesicle appeared thicker in comparison to that of the wild-type animals (Figs. 3E, 3F). By E14, transgenic eyes



**FIGURE 2.** *Msx2* transgenic animals were microphthalmic. (A, B) The overt appearances of the nontransgenic optic vesicle (A) and its transgenic counterpart (B) at E11.5. Nontransgenic embryo showed a square-shaped optic vesicle outlined by the dark pigmentation of the RPE (arrow). In the *Msx2* transgenic embryo, the optic cup was clearly reduced in size and abnormally shaped (arrow). (C) At E14.5, the morphology of the embryonic eye of a nontransgenic embryo was well-defined (arrow). (D) The embryonic eye of a transgenic littermate appeared small (arrow).



**FIGURE 3.** Histomorphology of optic vesicles in *Msx2* transgenic embryos. (A) At E9.5, the optic diverticulum of the nontransgenic embryo had a typical columnar epithelium-like appearance (arrowhead) that was in close contact to the presumptive lens ectoderm. (B) In the transgenic embryo, the presumptive retinal neuroepithelium became thin and lost its usual rigidity (arrowhead). (C) At E10.5, after lens induction, the lens vesicle (arrowhead) formed as a result of an invagination process. (D) In the transgenic embryo, lens induction occurred, as demonstrated by the presence of a small abnormal lens-like structure (arrowhead). The entire optic cup appeared smaller in comparison to the wild-type optic vesicle in (C). (E) At E11.5, a well-defined optic stalk in this nontransgenic embryo served as morphologic landmark that divided dorsal and ventral halves of the neuroretina. Arrowhead: anterior lens vesicle. (F) In the transgenic optic vesicle, the lens failed to develop into a vesicle-like structure (arrowhead), fewer cells populated the neural retina, and the RPE cells were columnar and thicker in appearance. Cells with pyknotic nuclei were abundant in the retina and the lens vesicle. (G) At E14.5, differentiation of RGC has occurred in the nontransgenic neural retina as RGCs send axonal projections medially through the optic stalk. (H) In this *Msx2* transgenic littermate, neural retina and lens vesicle survived but was severely reduced in size (arrowhead). No trace of neural fiber was

were greatly reduced in size (Fig. 3H). All transgenic animals lacked an optic nerve as a consequence of either total absence of or very few surviving RGCs. Absence of neurofilament immunostaining in the nerve fiber layer of E14 transgenic retinas provided further support for the loss of RGCs (data not shown). This became more apparent in retinas of adult transgenic animals that carried the *Msx2* transgene (Fig. 3J). In addition to a sparsely populated RGC layer, a reduction in number of cells and the thickness of the outer and inner nuclear layers was apparent (Fig. 3J).

### Changes in Cell Proliferation

The loss of neural retinal cells in *Msx2* transgenic mice could be due to a reduction in cell proliferation or enhanced cell death or both. To demonstrate a change in cell proliferation, we performed *in vivo* BrdU labeling of embryos and performed histologic sections of embryonic eyes. At E9.5, the rate of BrdU incorporation in the optic primordia was not altered in transgenic animals in comparison with nontransgenic embryos (38.5% in *Msx2* transgenic embryos; 37.5% in control embryos;  $P > 0.05$ ; Fig. 4A, 4B, 4E). However, far fewer cells were present in the presumptive optic neuroepithelium of transgenic embryos. This reduction in retinal cell number became more apparent in optic vesicles of transgenic embryos at E11 (Fig. 4C), when a significant reduction in the number of BrdU-labeled cell nuclei in optic vesicles was seen (2.3% in *Msx2* transgenic embryos; 52.8% in control embryos;  $P < 0.001$ ; Figs. 4B-E).

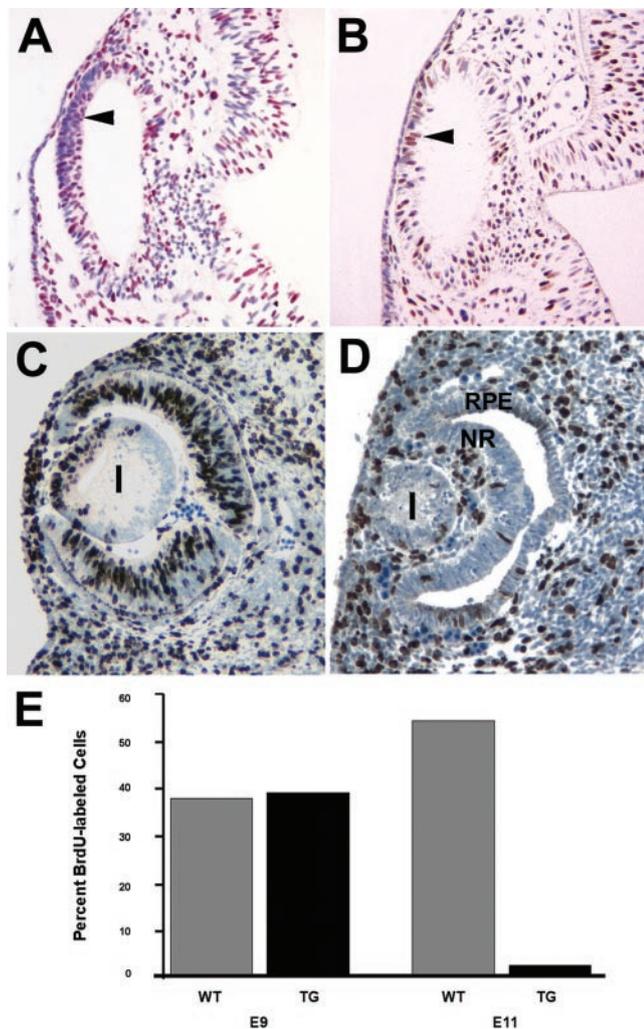
### Induction of Apoptosis

To determine whether the reduction in cell number in the presumptive retinal neuroepithelium is due to increasing PCD, we performed a TUNEL assay. We identified an increase in the number of cells undergoing DNA fragmentation in the presumptive neural retinas of *Msx2* transgenic animals as early as E9.5 (Fig. 5B), indicating that active apoptosis had taken place during the inductive phase of eye development. In contrast, very few if any apoptotic cells were detected in the presumptive neural retina of nontransgenic retinas, although a large number of apoptotic nuclei were present in the prospective optic stalk region in both transgenic and nontransgenic embryos (Figs. 5).

### Alteration in *Bmp4* and *Bmp7* Gene Expression

Although *Msx2* promoter activity was shown to be spatially restricted in the dorsal optic vesicle, overexpression of the *Msx2* transgene led to a global phenotypic change in the developing eye. We hypothesized that overexpression of *Msx2* may cause a generalized effect by perturbing the expression of diffusible factors that are known to be involved in early eye development. Ample experimental evidence has shown reciprocal regulation among BMP and *Msx* genes, and these in turn affect morphogenetic outcomes by promoting cell apoptosis.<sup>64-66</sup>

seen exiting the optic stalk. Mesenchymal cells invaded the vitreous space. The cornea failed to develop. (I) The wild-type adult retina was highly structured. Differentiated retinal cells resided in three specialized cell layers—the inner and outer nuclear layers and the RGC layer—with the RGC occupying the outermost layer and the photoreceptors populating the innermost layer. (J) In this *Msx2* transgenic retina, a large reduction in cell number among all three layers was evident. C, cornea; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; L, lens; NR, neuroretina; ONL, outer nuclear layer; OPL, outer plexiform layer; RPE, retinal pigmented epithelium; OS, optic stalk. Scale bar, 100  $\mu$ m.



**FIGURE 4.** Reduction in cell proliferation in the neural retina of the *Msx2* transgenic animals. To detect cells that underwent mitosis, BrdU was incorporated into the developing embryo through intraperitoneal injection of pregnant animals. Cell nuclei that incorporated BrdU were detected immunohistochemically, with a mouse monoclonal antibody. (A, B) At E9, proliferating cells (reddish) were found throughout the entire neural retina in the nontransgenic embryo (A) and the transgenic embryo (B). Although the percentage of cells incorporating BrdU appeared virtually identical between the nontransgenic and transgenic eye primordia, the total number of cells in the presumptive neuroretina was significantly reduced in the transgenic embryo. (C) At E11.5, more than 50% of retinal neuroblasts were actively dividing in the nontransgenic eye. (D) In the retina of a transgenic animal at E11.5, substantially fewer cells in the retina took up BrdU (dark brownish stain), although many cells that invaded vitreous space were actively proliferating. (E) Histogram of rate of cellular proliferation. At E9, close to 40% of neuroepithelial cells in the presumptive neuroretina were dividing in both transgenic (TG) and nontransgenic (WT) embryos. At E11.5, a dramatic reduction in proliferation rate is evident in transgenic eyes. Percentage of BrdU-labeled cells was calculated by dividing total number of BrdU-labeled cells by total number of cells in the entire neuroretina.

We therefore performed *in situ* hybridization to examine *Bmp4* and *Bmp7* gene expression in *Msx2* transgenic embryos. Unexpectedly, we observed a downregulation of *Bmp4* gene expression in the dorsal neural retina of the *Msx2* transgenic embryo at E9.5 and E10.5, although the level of *Bmp4* gene expression in the maxillary processes remained unchanged (Figs. 6A-H). This *Bmp4* expression domain corresponds to a region where many apoptotic nuclei have been detected in the

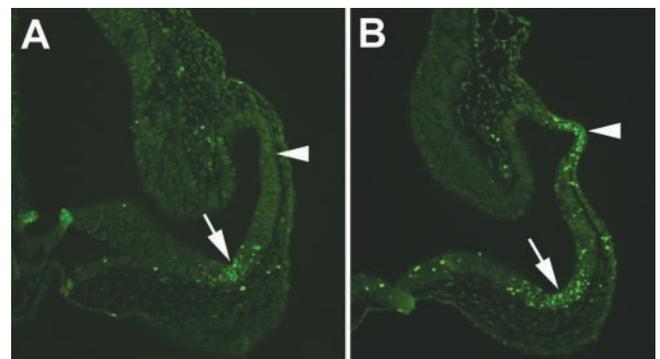
transgenic eye (Fig. 5B). In addition, the hybridization signal for *Bmp7* transcripts was more intense and broad in the transgenic eye, especially in the ventral retina indicating a generalized induction of *Bmp7* gene transcription (Figs. 6I-L).

### Changes in RPE Cell Markers in the Bilayered Eye Rudiment

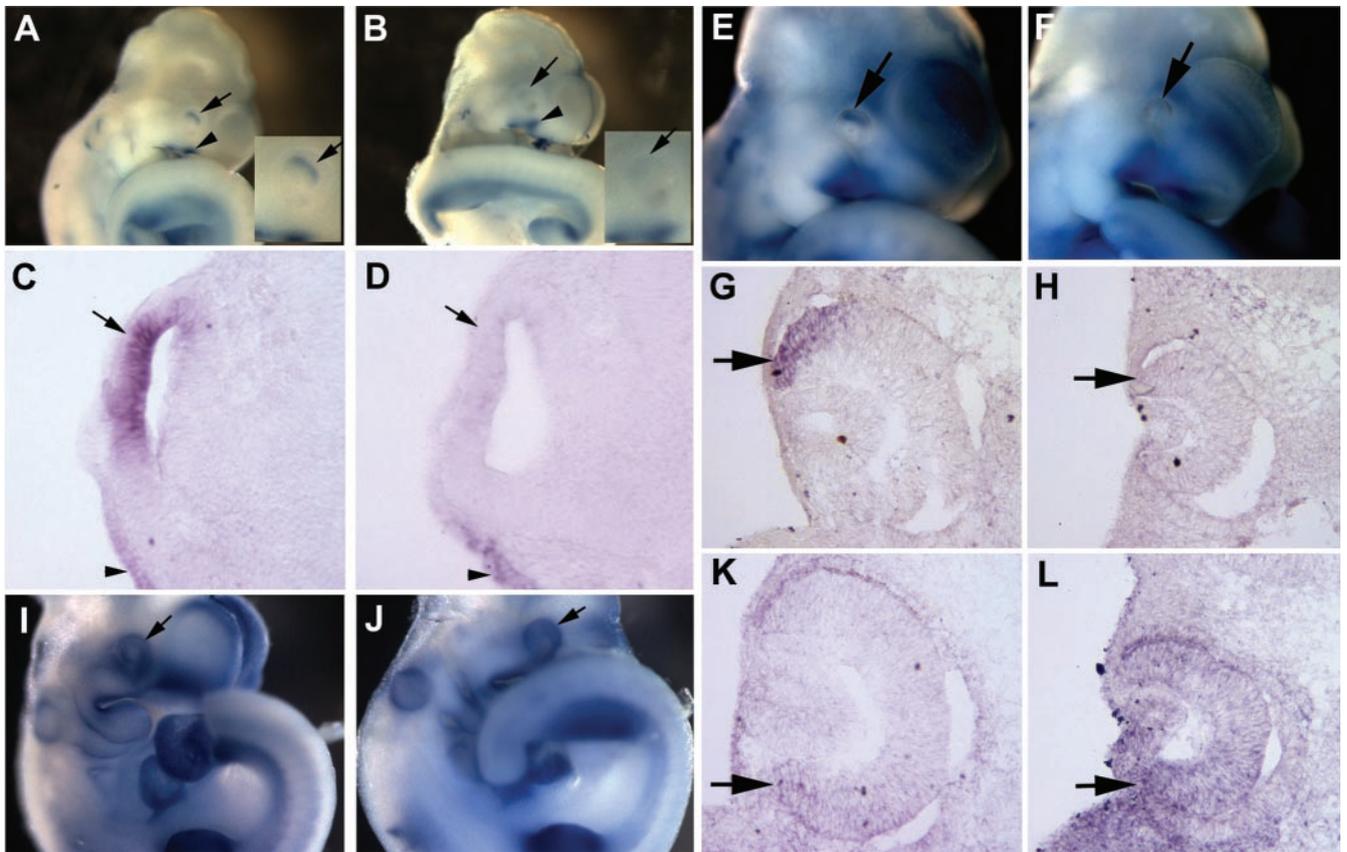
In *Msx2* transgenic animals that appeared anophthalmic, we were intrigued that the RPE survived *Msx2*-induced PCD and became a bilayered structure (Figs. 7A, 7B). To determine whether cells in the presumptive inner RPE layer indeed express the RPE phenotype, we examined the expression of genes that are predominantly expressed in RPE cells, including tyrosinase-related protein 2 (*Trp-2*) and connexin43 (*Cx43*).<sup>58,59</sup> *Trp-2* encodes the melanogenic enzyme DOPACHrome tautomerase (DT), which converts DOPACHrome to 5,6-dihydroxyindole carboxylic acid. It is one of the earliest expressed markers in RPE cells before the appearance of pigmented granules.<sup>58</sup> *Cx43* encodes a gap junction protein that is known to be important in intercellular communication and neural crest development.<sup>63</sup> In the developing eye, *Cx43* expression is spatially restricted to the RPE and the ciliary margin.<sup>58</sup> We hybridized 35S-labeled antisense RNA probes to sections of optic vesicles from *Msx2* transgenic and nontransgenic animals. In addition to detecting intense hybridization signals in the RPE and presumptive ciliary margin (Figs. 7E, 7G), we also observed intense expression of *Trp-2* in cells that populated the inner layer of the transgenic optic vesicle (Figs. 7F, 7H). We also observed an unexpected expansion in the expression domain of the *Cx43* in the inner layer of the optic vesicle in the *Msx2* transgenic animals (Figs. 7K, 7L). These *Cx43*-expressing cells were phenotypically similar to retinal neuroepithelium (Figs. 7I, 7J). *Pax6* expression, however, was not altered in transgenic retinas (data not shown).

### DISCUSSION

In the present study, we have shown that spatially restricted overexpression of the *Msx2* gene in transgenic animals led to microphthalmia and optic nerve aplasia. The temporal and spatial appearance of retinal apoptosis in the dorsal aspect of the eye primordium correlated with *Msx2* transgene expression. However, the apoptosis extended beyond this region to



**FIGURE 5.** *Msx2* transgenic animals showed an increase in apoptosis in the presumptive neural retina. (A) In the optic primordia of a nontransgenic animal at E9.5, apoptotic nuclei, as shown by bright TUNEL fluorescence was virtually absent in the neural retina (arrowhead), although many cells in the presumptive optic stalk were intensely labeled (arrow). (B) In the optic vesicle of the transgenic littermate, numerous cells in the neural retina were labeled as a result of PCD (arrowhead). TUNEL also stained cells in the presumptive optic stalk (arrow).



**FIGURE 6.** Suppression of *Bmp4* expression and induction of *Bmp7* expression in the neural retina of *Msx2* transgenic animals. (A) Wholemount in situ hybridization of an E9.5 wild-type embryo showed strong hybridization signal for *Bmp4* gene expression in the dorsal retina (arrow and inset). (B) Hybridization signal for *Bmp4* expression in the transgenic optic primordia was barely detectable (arrow and inset). Expression level in the maxillary process was not affected (A, B, arrowhead). (C, D) In situ hybridization showed strong *Bmp4* expression in the presumptive retinal neuroepithelium (arrow) of a wild-type embryo (C) and substantially reduced *Bmp4* expression in the transgenic embryo (D). (E, G) At E10.5, the level of *Bmp4* expression in the dorsal retina remained high (arrow). (F, H) *Bmp4* expression in the transgenic neural retina was barely detectable in the dorsal neural retina (arrow). (I) At E9.5, *Bmp7* expression was restricted to the presumptive RPE (arrow) and an inner ring of presumptive retinal neuroepithelial cells. (J) In the *Msx2* transgenic embryo, *Bmp7* expression was spread more widely in the dorsal aspect of the optic primordia (arrow). (K) At E10.5, *Bmp7* in the neuroretina of nontransgenic embryos was broadly but weakly expressed, although ventral retina appeared to produce more *Bmp7* transcripts (arrow). (L) In the transgenic retina, more intense *Bmp7* hybridization signal was observed. The hybridization signal in the ventral retina appeared to be more robust (arrow).

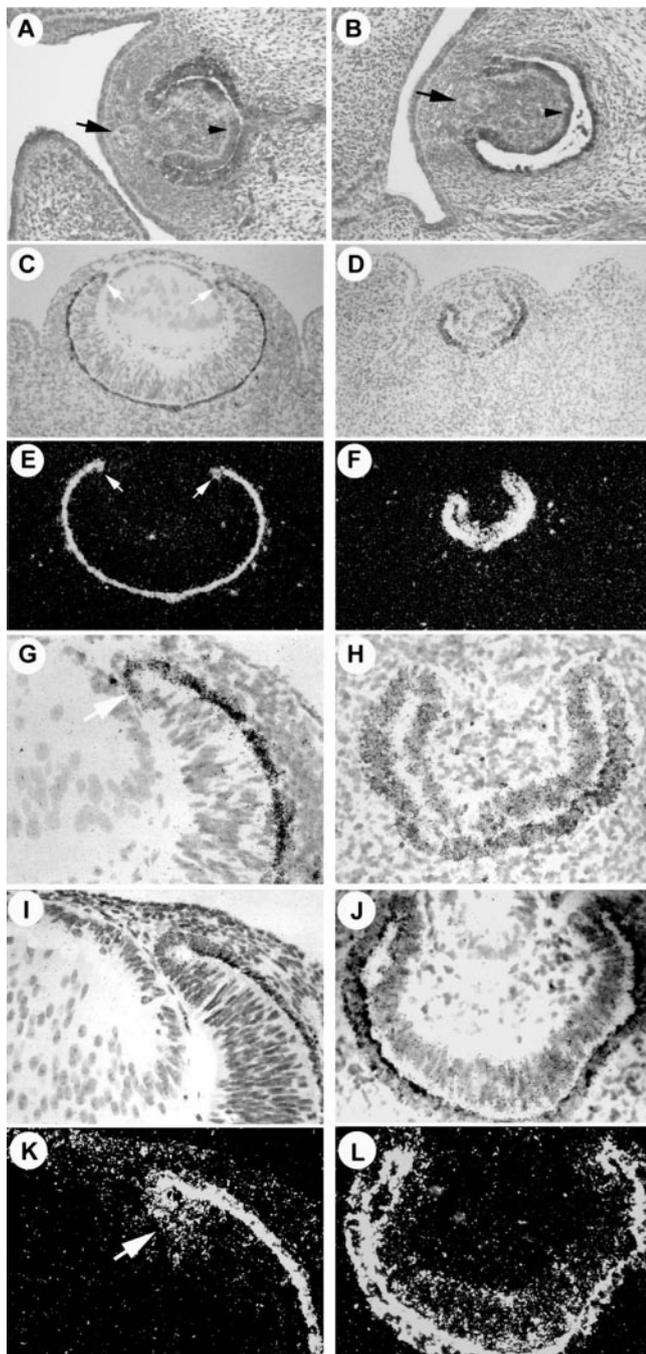
ventral retina. Because more than one transgenic line shows this phenotype, the retinal cell death is most likely due to the expression of the *Msx2* transgene rather than positional effects. The microphthalmic phenotype was accompanied by a significant decrease in *Bmp4* gene expression in the dorsal retina and an increase and broader distribution of *Bmp7* transcripts. Retinal apoptosis together with an overall reduction in proliferation resulted in the thinning of the retina and microphthalmia. In the most severely affected *Msx2* transgenic animals, this process occurred so rapidly that only a small fraction of the neural retina remained at E12, and by E14.5 the neural retina was not identifiable.

The reduction in proliferation in the neuroretina of the optic cup may be due to either a direct or indirect effect of *Msx2* overexpression. One possibility is that *Msx2* inhibits cell proliferation by perturbing gene expression of cyclins and cyclin inhibitors.<sup>23–26,75</sup> Alternatively, *Msx2* may suppress the function of these molecules by forming inactive protein-protein complexes.<sup>73,74</sup> *Msx2* is known to sequester transcriptional activators by forming inactive protein complexes to suppress gene expression.<sup>73,74</sup> In the chick limb bud, overexpression of *Msx2* has been shown to inhibit proliferation of limb mesenchyme, although the mechanism is not yet defined.<sup>55</sup> Another possibility is that induction of *Bmp7* expres-

sion may indirectly block proliferation in the *Msx2* transgenic retina. Overexpression of *Bmp7* in the lens has been shown to reduce proliferation in lens epithelial cells.<sup>36</sup>

Many studies have shown that *Msx2* can function as a regulator of apoptosis in developing vertebrate embryos. For example, the *talpid2* (*ta2*) chick limb mutant does not demonstrate cellular apoptosis in the anterior and posterior limb bud border and does not express *Msx2* in the limb mesoderm.<sup>64,65</sup> *Msx2* has been implicated in causing apoptosis in specific avian and murine neural crest populations.<sup>51,52</sup> Overexpression of *Msx2* induces apoptosis of mouse embryonic stem cell aggregates.<sup>54</sup> The proapoptotic activity of *Msx2* is apparently conserved evolutionarily. Recently, Mozer<sup>66</sup> has shown that in the fruit fly, overexpression of the *msb* gene, the *Drosophila* homologue of the mouse *Msx* genes, leads to photoreceptor apoptosis and the arrest of eye development.

How does a spatially restricted overexpression of the *Msx2* transgene cause a global defect in eye development? Whereas we have not defined the molecular basis for *Msx2*-induced apoptosis in the developing neuroretina; it has been shown that *Msx2* can mediate proapoptotic activities of BMP4.<sup>56</sup> One possibility is that *Msx2* overexpression invokes the canonical apoptotic pathway by altering gene expression of *Bax* and *Bad* or the *Bcl-2* family of genes leading to caspase activation.<sup>67,68</sup>



**FIGURE 7.** In situ hybridization demonstrating that cells in the bilayered eye rudiment expressed pigmented epithelial cell markers. (A) Histomorphology of a degenerated eye from a transgenic embryo at E12.4. The inner layer of the bilayered eye rudiment still showed the histomorphology of a neural retina (*arrowhead*). *Arrow*: remains of the lens vesicle. The vitreous body was invaded and replaced by a mixture of erythrocytes and mesenchymal cells. (B) At E14.5, the inner layer assumed the appearance of pigmented epithelium, as shown by pigment granules (*arrowhead*). The remains of the lens vesicle persisted (*arrow*). (C, E, G) Eye sections from wild-type embryos at E12 were hybridized with the *Trp2* antisense probe. Intensely bright hybridization signal viewed by dark-field microscopy (E) and appearing as very fine and discrete black silver grains under bright-field illumination (G) were visible only in the RPE cells and the future ciliary body (E, *arrow*). (D, F, H) In the *Msx2* transgenic littermate at E12, *Trp2* transcripts were localized to the inner layer of the eye rudiment. Note the abnormal thickness of the RPE layer in comparison with the wild-type RPE. (I, K) Eye sections from nontransgenic embryos at E12

Another possibility is that *Msx2* overexpression triggers a *p53*-dependent apoptotic response.<sup>69</sup> However, we can reasonably conclude that the loss of *Bmp4*-expressing cells in the dorsal aspects of the neural retina is due to *Msx2*-induced apoptosis through a cell-autonomous mechanism. Eyes with *Msx2* overexpression also showed increased *Bmp7* expression throughout the optic cup, suggesting an indirect effect of *Msx2*. Because increased *Bmp7* expression can also induce apoptosis,<sup>29,36,53</sup> this may explain the global retinal defect.

The importance of BMP signaling in the development of vertebrate optic vesicle has been shown repeatedly by gene inactivation and gain-of-function approaches in mice and avian systems. Together these studies demonstrated that the proper balance of BMP signaling is required for proper morphogenesis and differentiation of the retina, lens, and optic stalk.<sup>28,30–32,34–36,70–72</sup> Results in the present study provide further support for this general conclusion. Overexpression of *Msx2* suppressed *Bmp4* expression in the transgenic optic vesicle before lens induction, whereas *Bmp7* expression was upregulated. Results of TUNEL indicated that the loss of *Bmp4* expression may be due to induced apoptosis of *Bmp4*-expressing cells instead of transcriptional suppression.<sup>73,74</sup> Because the *Msx2* transgene is not expressed in the ventral portion of the optic vesicle, the increase in expression level and the dorsal expansion of the *Bmp7* expression domain is most likely an indirect effect of *Msx2* overexpression. We speculated that the induced *Bmp7* expression partially complements the loss of *Bmp4*, to allow lens induction to go forward and thus the formation of a small optic cup. Alternatively, *Msx2* may possess its own lens induction activity in the absence of BMP4 when it is expressed at appropriate levels. Furuta and Hogan<sup>28</sup> have shown that eye development in *Bmp4* null mutants was arrested before lens induction. *Msx2* expression was not detectable in the optic vesicles of the *Bmp4* null mutant. After the implantation of exogenous BMP4, *Msx2* expression was restored in the *Bmp4* null optic vesicle, and lens induction occurred.<sup>28</sup> In contrast to induction of *Bmp7* expression in the optic vesicle of *Msx2* transgenic embryo, *Bmp7* expression was not altered in the *Bmp4* null mutant optic vesicle. Recently, Hung et al.<sup>36</sup> have shown that lens specific ectopic expression of *Bmp7* can induce *Msx2* expression in the developing neuroretina. Together, these results suggest that *Msx2* and *Bmp7* indirectly regulates the expression of each other.

The conversion of ventral RPE into neuroepithelium by blocking BMP signaling in the developing optic vesicle with the BMP antagonist noggin<sup>70</sup> is in contrast to our finding of transdifferentiation of neuroretina into RPE-like cells in the *Msx2* transgenic eye, as demonstrated by the ectopic expression of the RPE markers *Trp-2* and *Cx43* in the *Msx2* transgenic neural retina. Recently, Holme et al.<sup>39</sup> demonstrated that forced expression of *Msx2* in dissociated chick RPE cultures induces neuronal marker expression. Together, these results lead us to believe that *Msx2* may have a function in resetting cell fate decisions and that BMP signaling may play an important role in these transdifferentiation processes.

In summary, maintenance of proper *Msx2* gene dosage and proper balance of BMP signaling is critical for normal eye morphogenesis. *Msx2* appears to play dual roles in promoting apoptosis and determining retinal fate. Future studies to elucidate the regulatory relationship between *Msx2* and BMP signals

were hybridized with the *Cx43* antisense probe. Intense hybridization signal was localized to the RPE layer, ciliary margin (K, *arrow*), and presumptive corneal ectoderm. (J, L) In the *Msx2* transgenic eye rudiment, *Cx43* hybridization signal spread to include the entire neural retina layer.

will advance our understanding of mammalian eye development and their role in retinal degeneration.

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