Microphthalmia Resulting from Msx2-Induced Apoptosis in the Optic Vesicle

Lan-Ying Wu,1,2 Min Li,2,3 David R. Hinton,4,5 Lin Guo,3 Shaoyun Jiang,3 Jian Tao Wang,3 Angie Zeng,3 Jian Bao Xie,5 Malcolm Snead,5 Charles Shuler,5 Robert E. Maxson Jr,1 and Yi-Hsin Liu3

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 specification.

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 arise. (Invest Ophthalmol Vis Sci. 2003;44:2404–2412) DOI: 10.1167/iovs.02-0317

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.1–3 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.4–7 Among these regulatory molecules are Pax6,5,6, Pax2,7 Six3 and Six6,8,9 Vax1,10 Mif1,11–13 Cx10,1,11–15 Lhx2,16 Rx,17 Otx1,18 Otx2,19–22 cyclin D1,23,24 p27kip1,25–27 BMP4,28–30 and BMP7.31–36 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 msh gene family37 has been shown to be expressed, though at low levels, in the developing vertebrate eye.38,39 Msx1;Msx2 double-null mutants showed arrest in eye development.40 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.41–47 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.46,47 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.42,43,44 A hypermorphic mutation in Msx2 is the cause of Boston type craniosynostosis.45 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.48,49 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.49 In eye development, apoptosis is thought to control cell number and ocular shape by eliminating excessive and abnormal cells. Most
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.36-38 Mx2 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.29,30,51-53 Ectopic expression of Mx2 has also been shown to induce apoptosis in a number of in vivo and in vitro systems.54-56

The present study provided evidence showing that Mx2 functions as a cell death-promoting factor in the developing murine optic vesicle. Deregulated expression of the Msx2 promotes cell death, and Msx2 functions as a cell death promoting factor in the developing neural retina, and initiate cellular apoptosis in the developing neural retina, and cause microphthalmia.

**Methods**

**Transgene Constructs**

A Spel–Notl fragment, which contains part of the first exon and minimal promoter of the Mx2 gene, was released from pBAD.6 and subcloned into pBluescript SKII (Stratagene, La Jolla, CA) to generate pSK-SN. Subsequently, a 5-kb XhoI–Spel fragment covering most of the upstream regulatory sequences for the Mx2 gene was subcloned into the Spel–XhoI site of pSK-SN to generate pSK-SNNX. The Notl–XhoI fragment from pSK-SNNX was then moved into Notl–XhoI sites of pGem11Zf (Promega, Madison, WI) to make pGem11Zf-NX-#3. To create the coding region for the transgene, a BshHII–XhoI fragment covering the entire coding region, intron, and 5′ sequences of the Mx2 gene was cloned into the HindIII–XhoI sites of pSP73 (Promega) to generate pCRII-T. A SalI–NdeI fragment that contains the entire first exon and intron was moved from pClone17 and replaced the KpnI–NdeI DNA fragment of pCRIIC-mx2. An NdeI site was regeneratized as a result. The intron was shortened to approximately 500 bp by removing a 3.1-kb KpnI fragment from this plasmid to generate pCRIIWT-KpnI. A PCR product containing the 5′ UTR and polyadenylation signal from the human Mx2 gene was then cloned into the EcoRI–XhoI site of pCRIIWT-KpnI to create pCRIIWT-KpnI%Hpa. To complete the construction of pMx2%T%Hpa, the SalI–Notl fragment in pCRIIWT-KnpI%Hpa was replaced by the SalI–Notl 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/6) x CBA/Jf1 females impregnated by (C57Bl/6) x CBA/Jf1 adult males (Jackson Laboratory, Bar Harbor, ME). Pseudopregnant females for embryo transfer were produced by matings between CD1 adult males (Jackson Laboratory, Bar Harbor, ME) and vasectomized CD1 adult females for embryo transfer were produced by matings between CD1 adult males (Charles River, Wil-lington, MA). Microinjection and oviduct transfer of injected zygotes was performed as described.44 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-amiopropirtriethoxysilane (TESPA)-treated slides (Sigma), and in situ hybridization was performed as described.45 35P-Labeled RNA probes were used at 5 × 10⁴ cpm/mL. Emulsion-coated slides were exposed for 12 to 14 days.

Probes for TRP were provided by Ian J. Jackson (Medical Research Council [MRC], Edinburgh, UK). The plasmids p5A and p5A5 were used to generate antisense and sense probes, respectively.50 Connexin43 probe was provided by Cecilia W. Low (University of Pennsylvania, Philadelphia, PA).59 Pax6 probe was obtained from Richard Maas (Harvard Medical School, Boston, MA).

**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 antibodies: rabbit polyclonal anti-neurofilament antibody (Sigma), polyclonal rabbit anti-Pax2 (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.44,61

**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× PBS (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.62 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 Mx2 Transgenic Animals**

Previously, we have identified a 5.2-kb promoter sequence in the Mx2 gene that, when fused to a lacZ reporter and an SV40
significantly in and around the optic placode was evident. (B) Which is destined to become RPE. (C) Nonradioactive in situ hybridization with the \textit{Msx2} riboprobe to demonstrate weak expression of the endogenous \textit{Msx2} gene in the developing optic vesicle of a non-transgenic 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 \textit{Msx2} transgenic embryo using \textit{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. The \textit{−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 \textit{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 non-transgenic embryos. In contrast to a barely detectable expression level of the endogenous \textit{Msx2} gene in the optic vesicle (Fig. 1C), the level of \textit{Msx2} transcript was increased significantly in the dorsal retina of the \textit{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 \textit{Msx2} transgenic animals from two separate transgenic lines had small eyes. All transgenic animals were born with body size and weight comparable to that of non-transgenic littermates. The extent of microphthalmos was not always bilaterally symmetrical. In one transgenic line, approximately 57% of \textit{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 \textit{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 1](image1.png)  
**Figure 1.** Expression of the \textit{Msx2−5.2/lacZ} transgene in the developing eye. (A) Wholemount β-galactosidase staining (blue) to monitor the \textit{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 \textit{Msx2} riboprobe to demonstrate weak expression of the endogenous \textit{Msx2} gene in the developing optic vesicle of a non-transgenic 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 \textit{Msx2} transgenic embryo using \textit{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.

![Figure 2](image2.png)  
**Figure 2.** \textit{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 \textit{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).
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. 3I). 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. 3I).

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 antibody staining on 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 be 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.

seen exiting the optic stalk. Mesenchymal cells invaded the vitreous space. The cornea failed to develop. (O) 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 RGCs 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 pigment epithelium; OS, optic stalk. Scale bar, 100 μm.
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 \(Msx2\) expression in the maxillary processes remained unchanged in the \(Bmp4\) embryo at E9.5 and E10.5, although the level of \(Bmp4\) gene expression in the dorsal neural retina of the \(Msx2\) transgenic animal led to an unexpected expansion in the expression of 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.

\(Msx2\) transgenic embryo (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).

**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 \(Msx2\) gene expression. However, the apoptosis extended beyond this region to

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

**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.

**Figure 5.** \(Msx2\) transgenic animals showed an increase in apoptosis in the presumptive neural retina. (A) In the optic primordium 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).
ventral retina. Because more than one transgenic line shows this phenotype, the retinal cell death is most likely due to the expression of the \( \text{Msx2} \) transgene rather than positional effects. The microphthalmic phenotype was accompanied by a significant decrease in \( \text{Bmp4} \) gene expression in the dorsal retina and an increase and broader distribution of \( \text{Bmp7} \) transcripts. Retinal apoptosis together with an overall reduction in proliferation resulted in the thinning of the retina and microphthalmia. In the most severely affected \( \text{Msx2} \) 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 had been reduced to a thin apical layer that 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 \( \text{Msx2} \) overexpression. One possibility is that \( \text{Msx2} \) inhibits cell proliferation by perturbing gene expression of cyclins and cyclin inhibitors.\(^{23-26,75,76} \) Alternatively, \( \text{Msx2} \) may suppress the function of these molecules by forming inactive protein-protein complexes.\(^{75,74} \) \( \text{Msx2} \) is known to sequester transcriptional activators by forming inactive protein complexes to suppress gene expression.\(^{75,74} \) In the chick limb bud, overexpression of \( \text{Msx2} \) has been shown to inhibit proliferation of limb mesenchyme, although the mechanism is not yet defined.\(^{55} \) Another possibility is that induction of \( \text{Bmp7} \) expression may indirectly block proliferation in the \( \text{Msx2} \) transgenic retina. Overexpression of \( \text{Bmp7} \) in the lens has been shown to reduce proliferation in lens epithelial cells.\(^{36} \)

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

How does a spatially restricted overexpression of the \( \text{Msx2} \) transgene cause a global defect in eye development? Whereas we have not defined the molecular basis for \( \text{Msx2} \)-induced apoptosis in the developing neuroretina; it has been shown that \( \text{Msx2} \) can mediate proapoptotic activities of BMP4.\(^{56} \) One possibility is that \( \text{Msx2} \) overexpression invokes the canonical apoptotic pathway by altering gene expression of \( \text{Bax} \) and \( \text{Bad} \) or the \( \text{Bcl-2} \) family of genes leading to caspase activation.\(^{57,64} \)
Another possibility is that Msx2 overexpression triggers a p53-dependent apoptotic response. However, we can reasonably conclude that the loss of Bmp4 expression may be due to induced apoptosis of Bmp4-expressing cells instead of transcriptional suppression. 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 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. In contrast to induction of Bmp7 expression in the optic vesicle of Msx2 transgenic embryonic, Bmp7 expression was not altered in the Bmp4 null mutant optic vesicle. Recently, Hung et al. 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 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. 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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