Pre-Clinical Research Report

Lint-specific deletion of the Msx2 gene increased apoptosis by enhancing the caspase-3/caspase-8 signaling pathway

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Abstract

Objective: To investigate the influence of Msx2 conditional gene knockout during lens development in mice.

Methods: Lens-specific Msx2 knockout mice were generated using the Cre-loxP system. The eyes of Msx2 conditional knockout (Msx2CKO) and wild-type (Msx2WT) mice were examined during embryonic and early postnatal periods using histological, immunofluorescence, in situ hybridization, cell proliferation, apoptosis, and mRNA microarray analyses.

Results: Msx2CKO mice exhibited small lens formation and microphthalmia after birth, while Msx2CKO embryos exhibited a persistent lens stalk, small lens formation, and microphthalmia. Conditional deletion of Msx2 also led to an increased apoptosis rate, a significant reduction in FoxE3 expression, and an upregulation of Prox1 expression in the lens vesicle during the early embryonic period. Microarray comparison of Msx2CKO and Msx2WT lens transcriptomes identified a large number of differentially expressed genes. Real-time PCR showed that Casp8 and Casp3 expression was upregulated in Msx2CKO mice at postnatal day 1.

Conclusion: The activation of apoptosis through the caspase-8/caspase-3 signaling pathway, together with the downregulation of FoxE3 expression, appeared to account for the smaller lens formation in Msx2CKO mice.

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Keywords
Msx2, apoptosis, FoxE3, lens development, Casp3, Casp8

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Introduction

The lens is a sensory organ that transmits and focuses light onto the retina. In vertebrates, the head surface of the embryo ectoderm develops into the lens.1–3 Homeobox genes function as essential transcriptional regulators in a variety of developmental processes.4 Msx genes encode members of the muscle segment homeobox gene family, including Msx1, Msx2, and Msx3, which are three unlinked members sharing 98% homology in their homeodomain.5 Msx2 is expressed at sites of epithelial–mesenchymal interaction during embryogenesis.6–13 Its encoded protein is both a transcriptional repressor and activator whose normal activity may establish a balance between survival and apoptosis, and could also promote cell growth under certain conditions.5,9,12,14,15

Previous evidence revealed that low levels of Msx2 expression were present in the developing murine optic vesicle,16,17 while Msx1/Msx2 double-null mutants showed arrested eye development.18 We previously demonstrated that Msx2 functions as an apoptosis-promoting factor in the developing murine optic vesicle; overexpression of the Msx2 transgene in mice resulted in optic nerve aplasia and microphthalmia.19 Germine knockouts (Msx2+/-) exhibited small lens formation or even aphakia, as well as a persistent lens stalk and iris hyperplasia in the anterior segment.14 Msx2+/- mice also previously showed retina folding and microphthalmia.14

Genetic evidence has indicated that lens formation depends on retinal formation in mice,20–23 and Pax6 was identified as a key regulator of early eye development. However, to further understand the Msx2 function in controlling embryonic lens development and to exclude the retinal influence on lens development through formation of the optic cup or gene expression in Msx2+/- mice, we produced Msx2 conditional knockout mice (Msx2CKO) using the Le-Cre mouse line.24 Comparative histological and gene expression analyses were performed to trace morphological and genetic changes and to identify molecular pathways associated with lens development.

Materials and methods

Animal model establishment

Mice homozygous for a floxed allele of Msx2 (Msx2fl/fl) were provided by Professor Benoît Robert (Pasteur Institute, Paris, France). Pax6 promoter-driven Cre transgenic mice (Le-Cre mice) were obtained from Professor David Beebe (Washington University, St, Louis, MO) with permission from Dr Peter Gruss (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany), and Professor Ruth Ashery-Padan (Tel Aviv University, Tel Aviv, Israel).24 ROSA26R mice were purchased from the Jackson laboratory.25 All mice were housed and cared for in accordance with the National Institutes of Health guide for the care and use of laboratory animals, and regulations set by the University of Southern California and China Medical University. The study protocol was approved by the ethics
committee of China Medical University (16005M).

Our mating scheme generated mice that were homozygous for the floxed allele (Msx2^floflo) and were either Le-Cre-positive (Le-Cre;Msx2^floflo;Msx2CKO) or Le-Cre-negative (Msx2^floflo, Msx2WT) as littermate controls (Figure 1a). Male mice carrying the Le-Cre allele were crossed with females carrying the R26R conditional reporter allele to generate Le-Cre;R26R embryos (time-staged embryos expressing a lacZ reporter gene). Genotyping was performed by PCR using primers for Cre recombinase (forward: 5'-TAATCGCCATCTTCCAGCAG-3' and reverse: 5'-CTCTGGTGATGATCTGCTGCTGAT-3') and floxed alleles of Msx2 (forward: 5'-GTTGAGCCGCAGTCTCCACCT-3' and reverse: 5'-GATTCCTGGGCGGCTTCTT-3'). PCR conditions to amplify Cre were: 94°C for 4 minutes, then 35 cycles of 94°C for 1 minute and 60°C for 1 minute, followed by 72°C for 1 minute, and 4°C for 10 minutes.

![Figure 1](image_url)

Figure 1. Generation of Msx2CKO mice. (a) Mating scheme. (b) Genotyping performed by PCR. The 350 bp band represents Le-Cre; the 347 bp band represents the wild-type; and the 381 bp band represents the mutant. (c) The recombination pattern of Le-Cre was detected by β-galactosidase whole mount staining at E10.5 and E14.5. (d) Whole mount in situ hybridization showing the absence of Msx2 mRNA in the Msx2CKO lens vesicle at E10.5 and E11.5. (e) Msx2CKO mice lacked eyelashes and hair on the surface of their eye lids and in a stripe running from the temporal to the nasal side of the eye (n≥3 per genotype) at P21. Msx2CKO mice showing reduced lens size and microphthalmia at P2. Histological sections of the Msx2CKO eyeballs showing reduction in the lens size, displacement of lens fiber nuclei toward the anterior and posterior of the lens, and vacuolation of cortical fiber cells at P21.
minutes. PCR conditions to amplify Msx2 were the same but amplification was carried out at 56°C rather than 60°C.

**RNA microarray analysis**

Total RNA was extracted from lenses isolated from Msx2CKO (n = 3) and Msx2WT mice (n = 3) on day P1 using an RNeasy micro kit (Qiagen GmbH, Hilden, Germany). Msx2WT lenses were used as controls. The microarray was carried out by Shanghai Biotechnology Corporation (Shanghai, China) using one-color microarray-based gene expression analysis. Microarray data and protocols have been submitted to NCBI’s Gene Expression Omnibus (GEO) database at http://www.ncbi.nlm.nih.gov/geo with a GEO accession number of GSE92947.

Differentially expressed genes (DEGs) were identified using online SBC Analysis System (SAS) statistical software (http://sas.shbio.com/). To identify the functions of DEGs, gene ontology (GO) and KEGG pathway enrichment analyses were carried out using online SAS software. Cytoscape ClueGO software (National Institute of General Medical Sciences, Bethesda, MD, USA) was used for DEG molecular function analysis.

**Real-time quantitative PCR of lens tissue**

RNA specimens were extracted from the lenses of three Msx2CKO and three Msx2WT mice at post-natal day (P)1 using the RNeasy micro kit (Cat#74004; QIAGEN GmBH). Quantitative PCR was carried out to verify the microarray results by amplifying Casp8 and Casp3 using primers forward: 5’-CCACGAGATTCTAGAAGGCTACCAAGGC-3’ and reverse: 5’-CTCACGTCATAGTTCACGCCAGTCA-3’, and forward: 5’-CTCACGTCATAGTTCACGCCAGTCA-3’, and forward: 5’-CCATGGTGAAGGGGTCATTATGGGACA-3’ and reverse: 5’-TGGACACAAATACACGGGATCTGTTTCTTTT-3’, respectively. PCR included SYBR Premix Ex Taq™ II (Takara, Dalian, China), and conditions were 94°C for 2 minutes, followed by 40 cycles of 94°C for 1 minute and 60°C for 40 s. Results were analyzed using the equation \( RQ = 2^{-\Delta\Delta CT} \).

**β-galactosidase whole mount staining**

Time-staged embryos expressing the lacZ reporter gene were fixed with 1% paraformaldehyde and stained at 37°C in the dark overnight by immersion in 1 mg/mL X-gal staining solution (https://www.jax.org/research-and-faculty/tools/cre-repository/whole-mount-staining-protocol). Whole mount in situ hybridization was performed using standard protocols. The Msx2 cDNA plasmid was purchased from the ATCC (Manassas, VA, USA). FoxE3 plasmids were kindly provided by Peter Carlsson (University of Gothenburg, Gothenburg, Sweden). Msx2 and FoxE3 RNA probes were labeled with digoxigenin-UTP according to the manufacturer’s recommendations (Roche, Basel, Switzerland).

**Whole mount in situ hybridization**

Time-staged embryos were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline. Whole-mount in situ hybridization was performed using standard protocols. The Msx2 cDNA plasmid was purchased from the ATCC (Manassas, VA, USA). FoxE3 plasmids were kindly provided by Peter Carlsson (University of Gothenburg, Gothenburg, Sweden). Msx2 and FoxE3 RNA probes were labeled with digoxigenin-UTP according to the manufacturer’s recommendations (Roche, Basel, Switzerland).

**Histological analysis**

Embryos between E9.5 to E16.5 and eyeballs at P2 and P21 were fixed overnight in Davidson’s fixative solution (glacial acetic acid:95% ethyl alcohol:10% neutral buffered formalin: distilled water 1:3:2:3). They were then dehydrated through a graded alcohol series, cleared in xylene, and embedded in paraffin. Sections were cut to 5 μm, then stained with hematoxylin and eosin. Lens size was quantified by measuring the anteroposterior and horizontal
diameters in both groups from E9.5 to E12.5.

Immunohistochemistry

After deparaffinization and rehydration, sections were boiled for 10 minutes in antigen repair solution, then blocked with 5% bovine serum albumin for 1 hour. They were incubated with the primary antibodies anti-Sox2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Pax6 (University of Southern California, Los Angeles, CA), anti-AP2-α (University of Southern California), and anti-N-cadherin and anti-E-cadherin (kindly provided by Dr Yue Zhao, China Medical University). Sections were then incubated with fluorescent-labeled secondary antibodies DyLight™ 549-conjugated donkey anti-mouse IgG and DyLight™ 488-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at room temperature for 2 hours. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole. Sections (5 μm) were mounted and viewed under a fluorescence microscope (Zeiss, Gottingen, Germany).

5-bromo-2’-deoxyuridine (BrdU) staining

Pregnant mice were sacrificed at various time points after conception. One h before sacrifice, the mice were injected intraperitoneally with 100 μg BrdU (Sigma, St. Louis, MO, USA) per gram of body weight. A BrdU kit was used (Roche), and cell counting was conducted according to a modified protocol.¹⁴

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

Apoptotic cells were detected using the fluorescein in situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN, USA).²⁹ Labeled cells were visualized with a fluorescence microscope and images were captured.

Statistical analyses

Statistical evaluations between control and mutant samples were performed using one-way analysis of variance. A P-value < 0.05 indicated that the observed differences were statistically significant.

Results

Rise in small lens formation from the conditional deletion of Msx2

We generated Msx2CKO and Msx2WT mice, and genotyped them using PCR (Figure 1a, 1b). The temporal and spatial activity of Le-Cre was characterized by whole mount β-galactosidase staining (Figure 1c).²⁵ We detected Msx2 transcripts by whole mount in situ hybridization beginning on E9 (data not shown). As early as E10.5, the Msx2CKO lens placode failed to detect any signals, suggesting that Msx2 lost its function at this time. A weak hybridization signal for Msx2 was observed in the dorsal retina and lens vesicle in Msx2WT mice (Figure 1d). The Msx2CKO lens placode also failed to detect any signals in the lens vesicle stage (E11.5) (Figure 1d). Noteworthy differences were observed between Msx2CKO and their wild-type littermates. At E9.5, no significant difference was observed between Msx2CKO and their wild-type littermates. At E9.5, no significant difference was observed between
the developing eyes of \(MSX2\)CKO and \(MSX2\)WT embryos (Figure 2a). By contrast, defects in the lens were evident as early as E10 and E10.5. At this stage, the lens vesicle was considerably smaller in \(MSX2\)CKO embryos (Figure 2a). At E11.5, the lens vesicle appeared smaller and was surrounded by neurilemma cells in the \(MSX2\)CKO group (Figure 2a). At E12.5, the lens vesicle epithelium was completely separated from

![Figure 2](image-url)

**Figure 2.** (a) HE staining. At E9.5, the optic vesicle (OV) is in contact with the lens placode and no structural differences are observed between \(MSX2\)CKO and \(MSX2\)WT embryos. At E10.5, the lens vesicle that had invaginated into the optic cup is considerably larger in the developing eyes of \(MSX2\)WT mice. At E11.5, the lens vesicle has closed in the eyes of both \(MSX2\)WT and \(MSX2\)CKO embryos, but appears smaller in \(MSX2\)CKO embryo. At E12.5, the epithelium of the lens vesicle has completely separated from the surface ectoderm in \(MSX2\)WT mice, but remains adherent in \(MSX2\)CKO mice. Black arrow: lens stalk. Re, Retina; Le, Lens; Co, Cornea. OV, optic vesicle. (b) Column diagram of the horizontal lens diameter (left) and anteroposterior diameter (right) of \(MSX2\)CKO and \(MSX2\)WT mice between E9 and E12.5. Significant differences from E10.5 to E12.5 were observed between the two groups \((P<0.05)\). (c) Altered cell proliferation. At E10.5, no differences were observed in the ratio of BrdU-positive lens cells between \(MSX2\)CKO and \(MSX2\)WT mice. At E12.5, the absolute number of lens epithelia incorporating BrdU was significantly reduced in \(MSX2\)CKO mice compared with \(MSX2\)WT mice, but the ratio of BrdU-positive cells was virtually identical. (d) At E14.5, the BrdU labeling rate in lens epithelial cells was the lowest in the 0° to 15° sector in both \(MSX2\)CKO and the \(MSX2\)WT mice. The ratio of BrdU-positive cells in the 60°–90° sectors was significantly increased in central lens epithelial cells in \(MSX2\)CKO mice compared with \(MSX2\)WT mice; no difference was found in the 15°–45° sectors in \(MSX2\)CKO mice compared with \(MSX2\)WT mice. (e) Cell counting method. Schematic of the division of lens epithelia (LE) into 15 sections showing the location of the transition zone (0°–15°) and the germinative zone (15°–30°) of the LE and the lens fiber (LF) compartment.
the surface ectoderm in Msx2WT embryos, while it remained adherent in Msx2CKO mice (Figure 2a). Subsequently at E14.5 and E16.5, Msx2CKO embryo eyes were greatly reduced in size and the cornea and lens had not separated, whereas the lens vehicle had completely separated from the surface ectoderm of Msx2WT littermates, and epithelial cells at the equator of the lens were disorganized (Figure S1). Significant differences in the horizontal lens diameter and anteroposterior diameter were observed between the two groups from E10.5 to E12.5 (n = 6, P < 0.05), but not at E9.5 and E10 (n = 6) (Figure 2b).

Changes in cell proliferation

To assess whether cell proliferation was involved in small lens formation, embryos were examined using in vivo BrdU labeling (Figure 2c, 2e). At E10.5, no differences were observed in the ratio of BrdU-positive cells between Msx2CKO and Msx2WT mice (n = 4). At E12.5, Msx2CKO mice showed smaller lens vesicles, and the absolute number of lens cells that incorporated BrdU was significantly reduced compared with wild-type littermates (P < 0.05), but the ratio of BrdU-positive cells was virtually identical (n = 4). We also evaluated the proliferation rate at E14.5, a relatively late stage of lens development when cells begin to withdraw from the cell cycle in preparation for fiber cell terminal differentiation. The BrdU labeling rate in lens epithelial cells was lowest in the 0° to 15° sector in both Msx2CKO and Msx2WT mice, with no significant difference between the two groups at E14.5 (Figure 2d). The ratio of BrdU-positive cells in 60°–90° sectors was found to be significantly increased in central lens epithelial cells at E14.5 in Msx2CKO mice compared with Msx2WT mice (n = 4, P < 0.01); no significant difference was found in 15°–45° sectors at E14.5 in Msx2CKO mice compared with Msx2WT mice (n = 4) (Figure 2d).

Increased apoptosis

At E10.5, a significantly higher level of lens epithelial cell (LEC) apoptosis was observed in Msx2CKO embryos that was mainly concentrated near the lens stalk (n = 4, P < 0.05) (Figure 3a). From E12.5 to E16.5, very few apoptotic LECs were observed in Msx2WT mice, but the absolute number of apoptotic LECs was significantly increased in Msx2CKO mice compared with Msx2WT littermates (n = 4, P < 0.01) (Figure 3b, 3c). Additionally, our in vitro study found that small interfering RNA knockdown of Msx2 in cultured murine α-TN4 cells increased the apoptosis rate of murine LECs in by over 3-fold (data not shown).

Enhanced Casp3/Casp8 signaling pathway affecting lens development

Microarray analysis showed there were 3412 DEGs with a greater than 2-fold change in expression in the Msx2CKO lens compared with the Msx2WT lens; of these, 1815 genes were upregulated and 1597 were downregulated (Figure 4a). Members of apoptosis signaling pathways, caspase 3 (Casp3) and caspase 8 (Casp8), were shown to be upregulated in the Msx2CKO lens compared with the Msx2WT lens (Figure 4a). KEGG pathway enrichment analysis, GO enrichment analysis, and molecular function ClueGO analysis were performed (Figure S2).

Real-time quantitative PCR was performed to detect the expression levels of apoptosis-related markers at P1; the expression of Casp3 and Casp8 was significantly increased in Msx2CKO lenses compared with Msx2WT controls (P < 0.05; Figure 4b).
Alterations in FoxE3 and Prox1 expression in Msx2CKO mice

The intensity and distribution of Pax6, Sox2, and AP2α transcription factors in Msx2CKO embryos were identical to their wild-type counterparts at E10.5 (Figure S3). Whole mount in situ hybridization revealed a dramatic reduction in FoxE3 expression at E10.5 in the lens pit of Msx2CKO embryos compared with wild-type littermates (Figure 4c). At E11.5, FoxE3 expression in the anterior lens epithelium of Msx2CKO embryos remained lower than in wild-type littermates (Figure 4d). Immunostaining of Prox1 expression was significantly increased in the lens vehicles of Msx2CKO embryos compared with wild-type littermates at E11.5 (Figure S3). At E14.5, the intensity of N-cadherin and E-cadherin expression in the anterior lens epithelium was identical in Msx2CKO and Msx2WT embryos (Figure S3).

Discussion

In this study, we observed that Msx2CKO mice exhibited small lens formation, lens stalk persistence, displaced lens fiber nuclei, and vacuoles in their cortical fiber cells compared with WT littermates. These abnormal phenotypes are similar to those observed in Pax6Sey/+, Sox11−/−, FoxE3+/−, and Msx2−/− mice. Microarray analysis and real-time PCR provided evidence that Casp3/Casp8 are activated and that the apoptosis caspase signaling pathway might participate in lens apoptosis of Msx2CKO mice.

Whether the lens formation depends on retina formation is one of the oldest questions in ocular development.

Figure 3. (a) At E10.5, a significantly higher level of lens epithelial cell apoptosis was observed that was mainly concentrated near the lens stalk in Msx2CKO embryos. (b, c) From E12.5 to E16.5, very few apoptotic lens epithelial cells were observed in Msx2WT mice, but the absolute number of lens epithelial cells undergoing apoptosis was significantly increased in Msx2CKO mice compared with Msx2WT mice.
mutants have previously shown varying degrees of microphthalmia with small lens formation or even aphakia and compromised laminar structure of the retina in severe cases. Additionally, both germline Msx2 knockouts and conditional Msx2 knockouts exhibited small lens formation, while ocular phenotypes of germline knockouts were more severe; this can likely be explained by the different timings of Msx2 deletions. Different degrees of eye abnormalities in Msx2CKO could be caused by the timing of Cre induction (i.e., early vs. late) or the absolute level of Cre expression. The effective timing and dosage of Cre may also disrupt Msx2 expression and downregulate FoxE3 mRNA levels to different extents.

During the early stages of lens development in the present study (E9.5–E12.5), Msx2CKO mice showed small lens pits and lens vesicles, which may reflect reduced cellular proliferation and/or enhanced cell death. Msx2, Bmp4, and Bmp7 regulate cell death during development by inducing the apoptosis of vertebrate retinal cells, neuronal precursors, and cranial neural crest cells. In Msx2CKO embryos, the BrdU incorporation rate in central epithelia was significantly increased compared with Msx2WT littermates at E14.5. Notably enhanced apoptotic activity was
also observed from E10.5 in Msx2CKO embryos compared with Msx2WT littermates.

Microarray analysis identified DEGs in the Msx2CKO lens, and Casp3 and Casp8 were found to be significantly upregulated in the Msx2CKO lens at P1. An increase in Casp8 activity could lead to stimulation of downstream Casp3, which subsequently activates the molecular cascade of apoptosis in LECs. Our data together indicate that conditional deletion of Msx2 in the lens induced apoptosis via the activation of Casp3/Casp8 apoptosis signaling. However, our research was limited to microarray profiling of whole lens tissue at P1; gene expression profiling of early embryonic stages awaits further investigation.

In the present study, immunostaining revealed that Sox2, Pax6, and Ap2-a expression patterns were the same as those reported earlier in the developing mouse eye (Figure S3). However, FoxE3 expression was significantly reduced (Figure 4c, 4d) and Prox1 expression was upregulated in the lens vesicles of Msx2CKO mice (Figure S3). Prox1 was previously shown to function downstream of FoxE3 and its expression was suppressed by FoxE3. Therefore, Msx2 must be either parallel to or downstream from Pax6, Sox2, and Ap2-a, and to control FoxE3 indirectly, and suppress Prox1 either directly or indirectly.

Another abnormal feature of Msx2CKO mice is the lens stalk (corresponding to Peters anomaly in humans), resulting from failed separation of the cornea and the anterior lens epithelium. FoxE3 is involved in this process and is expressed in the early lens placode around E9 until it is turned off in differentiating primary lens fibers at E12.5. In FoxE3+/− mice, the lens vesicle does not close and the lens remains irregular in shape and size with a persisting lens stalk. The lack of separation may affect the distribution but not the intensity of cell adhesion molecules in the anterior lens epithelium, such as E- and N-cadherins. This was observed in our current study in that the intensity of two adhesion molecules was identical between Msx2CKO and Msx2WT mice at E14.5. Moreover, nuclei displacement in lens fiber cells during lens differentiation can be explained by the involvement of FoxE3 expression regulating the DnaseII-like acid Dnase (Dlad). In FoxE3 null mice, Dlad is significantly downregulated, resulting in cataract.

In conclusion, the increased programmed cell death and cell proliferation alteration in the mouse lens may contribute to disorganized lens formation, resulting in microphthalmia in Msx2CKO mice. Casp3 and Casp8 are candidate signaling molecules regulating lens development in mice, and Msx2 appears to function as an integrator in these signaling pathways, playing a crucial role during early lens development.

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