Developmental analysis of the eye lens obsolescence (Elo) gene in the mouse: cell proliferation and Elo gene expression in the aggregation chimera

ATSUSHI YOSHIKI1,2, MAKOTO HANAZONO2, SEN-ICHI ODA3, NOBORU WAKASUGI4, TERUYO SAKAKURA2 and MORIAKI KUSAKABE2,*

1Laboratory of Animal Genetics, Faculty of Agriculture, Nagoya University, Chikusa, Nagoya 464, Japan
2Laboratory of Cell Biology, RIKEN, Tsukuba Life Science Center, 3-1-1 Koyadai, Tsukuba Ibaraki 305, Japan
3Research Institute of Environmental Medicine, Nagoya University, Chikusa, Nagoya 464, Japan
4Laboratory of Animal Reproduction, Faculty of Agriculture, Nagoya University, Chikusa, Nagoya, 464, Japan

* Author for correspondence

Summary

This study investigates the primary effect of the eye lens obsolescence (Elo) gene of the mouse. Morphological features of the Elo lens were defined as follows: (1) deficient elongation of lens fiber cells, (2) morphological abnormality of nuclei of lens fiber cells, (3) lack of eosinophilic granules in the central fiber cells and (4) rupture of lens capsule in the posterior region. We have immunohistologically examined, by means of an in vivo BrdU incorporation system, whether or not the Elo gene regulates cell proliferation during lens development. The lens fiber cells were morphologically abnormal in day 13 embryonic Elo lens. However, there were no significant differences in morphology or cell proliferation between normal and Elo lens epithelium until day 14 of gestation. After day 15, the total cell number in the Elo lens epithelium was significantly less than that in the normal, but the total numbers of S-phase cells in the two genotypes were not significantly different. The ratio of the total S-phase cell number to the total number of lens epithelial cells may be affected by the developmental stage, but not directly by the genotype. The genotype, however, may be having a direct influence at later ages because malformation of Elo lens fiber cells must cause reduction of the total number of lens epithelial cells in older embryos. Although, at 30 days old, Elo lens cells were externally extruded through the ruptured capsule into the vitreous cavity, BrdU-labelled lens epithelial cells were detectable. To investigate whether the Elo lens phenotype is determined by its own genotype or by its cellular environment, we produced aggregation chimeras between C3H-Elo/+ (C/Q) and BALB/c (c/c). Most lenses of BALB/c dominant chimeras were oval in shape without the ruptured lens capsule. However, they were opaque in the center and slightly smaller in size than normal. The lenses of C3H-Elo/+ dominant chimeras were morphologically similar to the Elo lens. Although normal nuclei were regularly arranged in the anterior region, Elo-type nuclei were located in the posterior region. Immunohistological staining by using anti-C3H strain-specific antibody demonstrated that the lens fiber cells with abnormal nuclei were derived only from C3H-Elo/+, not from BALB/c. These observations suggest that the primary effect of the Elo gene in the developing lens may be specific to the fiber cell differentiation rather than to the cell proliferation. Moreover, the Elo gene may be autonomously expressed in the differentiating lens fiber cells, and intracellularly inhibit fiber cell elongation.

Key words: Elo, lens development, BrdU, immunohistochemistry, aggregation chimeras, lens fiber differentiation, lens capsule, anti-C3H strain-specific antigen antibody.

Introduction

The vertebrate lens is composed of two distinct cell types: (1) fiber cells that make up the bulk of the lens; and (2) monolayered epithelial cells that cover the anterior surface of the fiber cells. Development and growth of the lens depends on proliferation of the epithelial cells and their differentiation into lens fiber cells (Hanna and O'Brien, 1961; McAvoy, 1978a). Sequential morphological changes in the developing
To observe the distribution of C3H-Elo/+ in situ.

Cells lens phenotype is determined by its own genotype or by means of aggregation chimeras (Konyukov, 1987). This extracellular component is known to be important for both induction of lens differentiation and normal physiological function of the lens cells (Hendrix and Zwaan, 1974; Muggleton-Harris and Higbee, 1987).

In mice, several mutant genes have been known to influence lens development (Green, 1981). A series of experiments on eye mutant genes such as $f_{\text{i}}$, $o_{\text{r}}$, $a_{\text{k}}$ and $C_{\text{at}}^{\text{zw}}$ were undertaken to study the mechanisms of gene expression and its regulation in the ontogenesis by means of aggregation chimeras (Konyukov, 1987). Recently, a congenital cataract defect caused by the $C_{\text{at}}^{\text{zw}}$ gene was suppressed and rescued in chimeras formed by aggregation between mutant and normal 8-cell-stage embryos (Muggleton-Harris et al. 1987). None of these studies, however, had directly shown the spatial distribution of cells derived from both component strains, because of the lack of a suitable in situ cell marker. Therefore, little is known about the mechanism of the rescue at the cellular level, but microenvironment must be playing an important role in the control of the gene expression. To apply the chimeric system effectively to analysis in developmental genetics, an in situ strain-specific marker, in addition to suitable markers for cell proliferation and differentiation, is needed.

The eye lens obsolescence gene (Elo) of the mouse arose from a spontaneous mutation during brother-sister matings between C57BL/6Nga and Japanese fancy mouse strains. The abnormality has been shown previously to be due to an autosomal dominant gene with complete penetrance, located on chromosome 1 (Oda et al. 1980a). Initial pathological changes, including impairment of elongation of central fiber cells, were detected on day 12 of gestation. Thereafter, the lens continued to develop abnormally, resulting in a small deformed lens by the late fetal stage (Oda et al. 1980b).

This study was undertaken to investigate the primary effect of the dominant gene, Elo, on the lens development. The mouse carrying a single Elo gene (Elo+/+) was analyzed as follows. (1) Chronological studies of the Elo lens development were performed by using semi-thin methacrylate sections. (2) Cell proliferation was examined in the developing Elo lens. Detection of bromodeoxyuridine (BrdU) incorporated into DNA was used as marker of proliferating cells. (3) To ascertain whether the Elo lens phenotype is determined by its own genotype or by its cellular environment, we produced aggregation chimeras between 8-cell embryos of Elo and normal mice. Then, chimeraism in the embryonic eye tissues was immunohistologically analyzed by anti-C3H strain-specific antigen (CSA) antibody (Kusakabe et al. 1988) to observe the distribution of C3H-Elo/+ cells in situ.

### Materials and Methods

#### Experimental animals

The mouse strains used in this study were C3H/HeN-Elo/+; C3H/HeN and BALB/c. All of them have been maintained under SPF condition in the animal facility of Life Science Research Center at RIKEN. The C3H/HeN-Elo/+ strain has been established from a conventional outbred stock (Quinlan et al. 1987) after embryo transfer followed by successive introduction of the Elo+/+ gene to the C3H/HeN background (N7). Heterozygous mice (Elo+/+) made by mating Elo/+ males with +/+ females were used instead of homozygous (Elo/Elo). Thus, the effect of a single dosage of the mutant gene can be precisely described in comparison with the same littermates of +/+ genotype. Histological features and microphthalmic phenotype of the Elo/+ lens were comparable to those of Elo/Elo described previously (Oda et al. 1981a, b), except that the initial pathological change of the lens in homozygous mice can be detectable 0.5 day earlier than that in heterozygous (Oda, personal communication). The day of vaginal plug detection was counted as day 0 of gestation. Eyes were taken on days 11-15 and 18 of gestation, and at 30 and 60 days postnatal.

#### BrdU labeling and tissue processing

Pregnant mice were given 0.5 mg BrdU (Sigma) suspended in saline by intravenous injection. 2 h after injection, the animals were killed for histological processing. Embryonic eyes with surrounding tissues and the enucleated eyes from 30- and 60-day-old mice were fixed in ice-cold buffered 10% neutral formalin (0.1 M sodium phosphate buffer, pH 7.4) for 2 h, dehydrated through ethanol series and embedded in polyester wax (BDH) for immunohistochemistry (Kusakabe et al. 1984) or in methacryl resin (Technovit 7100, Kulzer) for light microscopy. Resin-embedded tissues were sectioned at 2 μm and stained with hematoxylin and eosin (HE). Wax-embedded tissues were serially sectioned at 4 μm. Sections were then adhered on the albumin-coated glass slides. Immunohistological staining procedure for BrdU has been described in detail previously (Hanazono et al. 1990).

#### Quantitative analysis of the influences of Elo gene on the developing lens epithelium

To evaluate the influences of Elo gene on the developing lens epithelium, both normal and Elo lenses were taken from days 13, 14, 15, and 18 embryos. Three lenses in each group were randomly selected and examined. Embedded lenses were sectioned at 4 μm thickness. Every fourth section in the center region (100 μm wide) was selected and the whole lens epithelial cell number counted in each section. The sum of each cell number in five sections was represented as a total cell number of individual lens epithelium. Neighboring sections were immunohistologically stained for the detection of BrdU-incorporated cells and S-phase cells in the lens epithelium were counted. The sum of each S-phase cell number in five sections was also represented as a total S-phase cell number of individual lens epithelium. The incidence of S-phase cells in each lens was calculated from the total cell number and the total S-phase cell number. These numbers of incidence in the data were transformed into angles, according to a formula of angular transformation. Influences of Elo gene on total cell numbers, total S-phase cell numbers in the developing lens epithelium and incidence of S-phase cells were analyzed by means of 2-way analysis of variance (2-way ANOVA), respectively.
Production of C3H/HeN-Elo/+→BALB/cA aggregation chimeras

C3H/HeN-Elo/+ embryos were obtained by mating C3H/HeN females with homozygous C3H/HeN-Elo/Elo males. Chimeras were produced by aggregating C3H/HeN-Elo/+ (Elo/+ , C/C) and BALB/cA (+/+ , c/c) embryos according to the method by Mintz (1973). Briefly, 4- to 8-cell embryos of two different genotypes were collected by flushing oviducts. Zona pellucidae were digested away with pronase (Boehringer Mannheim). Embryo aggregation was performed in microdrops of M2 medium (Hogan et al. 1986) containing 5 μl ml⁻¹ phytohemagglutinin-P (Difco) (Mintz et al. 1985). Aggregated embryos were cultured in M16 (Hogan et al. 1986) for 24 h at 37°C in an atmosphere of 5% CO₂ and 95% air. Then they were transferred into uteri of pseudopregnant C57BL/6J recipients. One group of recipients was killed during gestation to obtain chimeric embryos and another group was allowed to litter. Histological samples to analyze chimerism of the embryos were prepared as described below. The chimeras after birth were excised for morphological and histological examination.

Immunohistological staining of chimerism with anti-C3H strain-specific antigen (CSA) antibody

The procedure of tissue fixation and CSA immunostaining will be described in detail elsewhere (Yoshiki et al. in preparation). Briefly, embryos in utero were placed in 0.1 M sodium phosphate buffer (pH 7.4) and irradiated in a microwave oven (Bio-Rad microwave processor H2500) for 20 min with 60 % power level at 40°C as prefixation. Then, the embryos were postfixed in ice-cold 95% ethanol containing 1% acetic acid, dehydrated with 100% ethanol, embedded in polyester wax and sectioned at 4 μm. Sections were dewaxed, inactivated for an endogenous peroxidase activity, washed in TBS, blocked for endogenous biotin with avidin/biotin blocking solution (Vector). After washing in TBS, sections were incubated with biotinylated anti-CSA antibody. Then, sections were washed in TBS, incubated with horseradish-peroxidase-conjugated avidin (Zymed), washed again in TBS followed by immersing into diaminobenzidine (DAB) substrate solution. DAB reaction products were intensified by DAB/gold/silver amplification staining (Burns et al. 1985). The sections were counterstained with HE.

Results

Chronological observation of developing lens of normal(+/+) and Elo(Elo+)/+ mice

Day 12

Cells in the posterior region of the lens vesicle, which faced the optic cup (presumptive retina), began to elongate. Lens abnormalities were not detectable.

Day 13

Normal and mutant type embryos were distinguishable by morphology of the primary lens fiber cells. In lenses of +/+ embryos, posterior lens epithelial cells elongated to close the cavity of the lens. In contrast, in lenses of Elo/+ embryos, elongation of lens fiber cells was poor and the cavity of the lens still remained. Nuclei of the poorly elongated fiber cells were located in the posterior region of the cytoplasm, and dense bodies were noted at nuclear poles.

Day 14

The elongation of the lens fiber cells proceeded in normal lens, and eosinophilic granules were observed in the anterior part of the cytoplasm. In the Elo lens, some central fiber cells became globular-shaped and these cells were located in the central part of the lens. Eosinophilic granules were not detectable in the Elo lens.

Day 15

In the +/+ lens, nuclei of lens fiber cells were located in the anterior part of the cytoplasm. Eosinophilic granules were abundant in the central fiber cells (Fig. 1A). The granules were distributed along the anterior–posterior axis of the fiber cells. The granules were also detected in nuclei of the central fiber cells (Fig. 1B). In the Elo lens, lens fiber cells in the cortex region appeared to elongate normally. However, in the deep cortex and central regions, nuclei of the fiber cells were located in the posterior part of the lens (Fig. 1C). Globular-shaped lens cells occupied central and anterior regions (Fig. 1D). Dense bodies were conspicuous in nuclei of deep cortex fibers (Fig. 1E). Eosinophilic granules were rarely detected.

Day 18

The normal lens was oval-shaped and larger in size than the Elo lens (Fig. 1F). Nuclei of the central fiber cells were round and chromatin was beginning to disappear. Eosinophilic granules in the fiber cells were also present. The Elo lens was conical rather than oval (Fig. 1G). Nuclei of fiber cells remained localized in the posterior region of the lens. All lens capsule of the Elo was ruptured in the posterior region. Globular cells appeared to be extruded through the opening. Eosinophilic granules were not visible. In late fetal stages the lens epithelium in Elo lens apparently covered a much smaller area than that in the normal one. Quantitative comparison between the Elo and normal lens epithelium will be described later.

30 days old mice

The anterior portion of normal lens was composed of a monolayer of cuboidal epithelial cells (Fig. 2A). Eosinophilic granules observed in the central fiber cells in late fetal stages were not visible. Most nuclei of the fiber cells were also absent, but fiber cells located in the peripheral region were nucleated. The Elo lens was severely malformed (Fig. 2B). The lens capsule was ruptured in posterior region and an irregular-shaped and thick lens capsule was formed anteriorly. Lens epithelial cells and globular-shaped cells were displaced from their normal positions. Some large globular-shaped cells filled the anterior and posterior eye chamber. Elo lens epithelial cells were squamous in shape rather than cuboidal, and the regular arrangement of the cells was disorganized.

Cell proliferation in the developing lens of normal(+/+) and Elo(Elo+)/+ mice

Indirect immunofluorescent staining of BrdU-incorpor-
ated S-phase cells in the developing lens is shown in Fig. 3A–F. 

**Normal**
In the early lens vesicle of day 11 embryos, there were no major differences in fluorescence labeling in different locations of the lens vesicle (Fig. 3A). After differentiation of fiber cells on day 12, cell proliferation was restricted to the anterior lens epithelium (Fig. 3B). On days 13–15 and 18, proliferating cells were

---

**Fig. 1.** Longitudinal sections of developing lenses from C3H/HeN-+/+ and C3H/HeN-Elo/+ embryos. (A) +/+-lens at day 15 embryo. (B) High magnification of the day 15 +/+-lens. Note that eosinophilic granules appear abundantly in elongating fiber cells. (C) Elo/+ lens at day 15. Nuclei of elongating fiber cells are seen in the posterior region of the lens. (D,E) High magnification of the day 15 Elo/+ lens. (D) Globular cells are noted in the anterior region. Eosinophilic granules are not detected as in the normal. (E) The posterior region of the Elo lens. Note dense nuclear chromatin is margined to the nuclear pole. (E) +/+ lens at day 18 embryo. (F) Elo/+ lens at day 18. Note that globular lens cells are extruded from the posterior region of the lens (arrow). Tissues were embedded in methacryl resin, cut at 2 μm and stained with HE. Bars: (A,C) 100 μm, (B,D,E) 50 μm, (F,G) 200 μm.
distributed only in the lens epithelium from the equatorial region to the center of the epithelium (Fig. 3C). The germinative zone of the lens epithelium, in which the labeled cells were most frequently observed, was located just above the lens equator. At 30 and 60 days postnatal, a few epithelial cells in the vicinity of ciliary body had incorporated BrdU (Fig. 3D).

Elo

By day 13 of gestation, the distribution pattern of proliferating cells seemed to be similar to that of the normal lens. On days 15 and 18, a number of epithelial cells of the small deformed lens were also labeled (Fig. 3E). At 30 days, proliferating cells were found in the lens epithelium most frequently near the ciliary body (Fig. 3F). At 60 days, fewer cellular components were observed within the original lens area. The remaining lens epithelial cells continued to proliferate even at this stage.

Quantitative analysis of the influences of Elo gene on the developing lens epithelium

As revealed in Tables, influences of Elo gene on total cell number (Table 1), total S-phase cell number (Table 2) and incidence of S-phase cells (Table 3) in the developing lens epithelium of both genotypes were analyzed by means of 2-way ANOVA. As shown in Table 1 and Fig. 4A, both developmental stage and genotype significantly influence the total cell number during lens development ($P<0.001$). After day 14, the number of lens epithelial cells in normal embryos continues to increase with age. However, Elo lens epithelium achieves a plateau in number so the Elo gene appears to influence the increase of lens epithelial cells in number with aging. As a natural consequence, it is likely that the epithelium in the mutant covers a much smaller area than the normal type epithelium. As shown in Table 2 and Fig. 4B, S-phase cells significantly decreased in number with advanced age ($P<0.001$); however, there were no significant differences between total S-phase cell numbers in both genotypes. Elo gene appeared not to affect the number of S-phase cell in the lens epithelium. We have also compared the incidence of S-phase cells between the two genotypes. As shown in Table 3 and Fig. 4C, the ratio of the S-phase cells to total cells significantly decreased with aging ($P<0.001$), but the effect of Elo gene on the incidence of S-phase cells was not evidently revealed. However, by analysis of the interaction between developmental stage and genotype, it seems that genotype may occasionally affect the incidence of S-phase cells in Elo lens.

Table 1. Table of analysis of variance in total cell number of lens epithelial cells during normal and Elo lens development

<table>
<thead>
<tr>
<th>Factor</th>
<th>S.S.</th>
<th>d.f.</th>
<th>M.S.</th>
<th>F</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>148,837.5</td>
<td>1</td>
<td>148,837.5</td>
<td>639.4</td>
<td>$P&lt;0.001$</td>
</tr>
<tr>
<td>Dev. stage</td>
<td>433,841.8</td>
<td>3</td>
<td>144,613.9</td>
<td>621.2</td>
<td>$P&lt;0.001$</td>
</tr>
<tr>
<td>Interaction</td>
<td>211,328.5</td>
<td>3</td>
<td>70,442.8</td>
<td>302.6</td>
<td>$P&lt;0.001$</td>
</tr>
<tr>
<td>Residual</td>
<td>3,724.7</td>
<td>16</td>
<td>228.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>797,732.5</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

S.S., sum of squares; d.f., degrees of freedom; M.S., mean square; F, variance ratio; Sig., significantly different; Dev. stage, developmental stage.
Fig. 3. Immunohistological staining of BrdU labeling in the developing lens from +/+ and Elo/+. (A) Lens vesicle of day 11 embryo. BrdU incorporation is noted in all positions of lens epithelium. (B) Lens of day 12 embryo. +/+ and Elo/+ lenses were not distinguishable at this stage. Lens epithelial cells that face neural retina are elongated. These elongated fiber cells are not labeled. (C) +/+ lens of day 15 embryo. Number of nuclei are labeled in the epithelium just above the lens equator (le). (D) +/+ lens at 30 days. A lens epithelial cells near the ciliary body (cb) is labeled (arrow). (E) Elo/+ lens of day 15 embryo. Number of nuclei of the epithelium are labeled also in the deformed lens. (F) Elo/+ lens at 30 days. A few nuclei of the epithelium near the ciliary body (cb) are labeled (arrows). Bars: (A) 100 μm, (B,C,E) 200 μm, (D,F) 100 μm. Abbreviation: cp, lens capsule; el, extruded lens cells.

epithelium with aging ($P<0.01$). The mutant gene effect seems to depend on the developmental stage. Fig. 4C indicated that the difference on day 18 between normal and Elo was greatest.

Production of Elo/+, C/C++, c/c aggregation chimeras
In the present study, we obtained 75 chimeras from a total 210 aggregations between Elo/+, C/C and +/+. 
Cell-autonomous Elo gene expression in chimeric mouse lens

Table 2. Table of analysis of variance in total number of S-phase cells during normal and Elo lens development

<table>
<thead>
<tr>
<th>Factor</th>
<th>S.S.</th>
<th>d.f.</th>
<th>M.S.</th>
<th>F</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>322.7</td>
<td>1</td>
<td>322.7</td>
<td>1.99</td>
<td>Not sig.</td>
</tr>
<tr>
<td>Dev. stage</td>
<td>18796.8</td>
<td>3</td>
<td>6252.6</td>
<td>38.99</td>
<td>Sig. P&lt;0.001</td>
</tr>
<tr>
<td>Interaction</td>
<td>532.3</td>
<td>3</td>
<td>177.4</td>
<td>1.09</td>
<td>Not sig.</td>
</tr>
<tr>
<td>Residual</td>
<td>2596.0</td>
<td>16</td>
<td>162.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>22427.8</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

S.S., sum of squares; d.f., degrees of freedom; M.S., mean square; F, variance ratio; Not sig., not significantly different (P>0.05); Sig., significantly different; Dev. stage, developmental stage.

Table 3. Table of analysis of variance in the incidence of S-phase cells during normal and Elo lens development*

<table>
<thead>
<tr>
<th>Factor</th>
<th>S.S.</th>
<th>d.f.</th>
<th>M.S.</th>
<th>F</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dev. stage</td>
<td>827.6</td>
<td>3</td>
<td>275.9</td>
<td>129.2</td>
<td>Sig. P&lt;0.001</td>
</tr>
<tr>
<td>Interaction</td>
<td>38.2</td>
<td>3</td>
<td>12.7</td>
<td>5.96</td>
<td>Sig. P&lt;0.01</td>
</tr>
<tr>
<td>Residual</td>
<td>34.2</td>
<td>16</td>
<td>2.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>909.6</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The analysis on the data transformed into angles.
S.S., sum of squares; d.f., degrees of freedom; M.S., mean square; F, variance ratio; Sig., significantly different; Dev. stage, developmental stage.

---

Fig. 4. Development of the normal and Elo lens epithelium. (A) Total number of the developing lens epithelial cells, (B) total number of the S-phase cells in the developing lens epithelium, (C) incidence of S-phase cells during lens development. Black bar, normal lens; white bar, Elo lens.

---

Fig. 5A. Development of the normal and Elo lens epithelium. C/4- to 8-cell embryos. These chimeras were analyzed at consecutive developmental stages. The number of chimeras studied at each stage is shown in Table 4.

Morphological and histological observation of the chimeras

Coat color variation and eye phenotype of 25 chimeras at 15 and 30 days of age are summarized in Table 5. Of the 25 chimeric mice, 7 had apparent microphthalmic eyes bilaterally. The lenses of these microphthalmic eyes were posteriorly ruptured and severely malformed (Fig. 5A). Lenses of the other 15 chimeras were oval in shape and only slightly smaller than normal lens. The lens capsule seemed to form normally. However, these lenses were opaque in the center as shown in Fig. 5B. Two chimeras had an opaque lens on one side and an Elo-type lens on the other. Of the remainder, one chimera had a normal-sized, clear lens in the left eye (Fig. 5C) and a opaque lens in the right. These data indicate that the lens of mutant mice can not be made normal by forming chimeras with normal mice.

Histologically, the lenses from microphthalmic eyes were similar to those of the Elo mice (Fig. 6A). The opaque lenses had a normal anterior lens epithelium and lens fiber cells in the cortex (Fig. 6B). The central part of the opaque lens could not be viewed, because tissue samples were too brittle for sectioning. In the central region of the lens of days 14–18 chimeric embryos and newborn chimeras, nuclei with normal
Table 4. Production of C3H-Elo/+↔BALB/c aggregation chimeras

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of aggregations transferred</th>
<th>Total (%)</th>
<th>Day of gestation/Day after birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elo/+, C/C++</td>
<td>210 (14)*</td>
<td>75 (35.7)</td>
<td>8 21 2 6 / 13 5 20</td>
</tr>
<tr>
<td>+/+, c/c</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*: Number in parentheses indicates the number of pregnant recipients.

Table 5. Coat color variation and eye phenotype of the C3H-Elo/+↔BALB/c chimeras

<table>
<thead>
<tr>
<th>Group</th>
<th>Coat color ratio</th>
<th>C/C: c/c</th>
<th>No. of chimeras with each phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>&gt;</td>
<td>2</td>
<td>Mic 0 1 0 0 Nor 3</td>
</tr>
<tr>
<td>2.</td>
<td>=</td>
<td>3</td>
<td>Mic/Op 2 3 0 0 Nor 8</td>
</tr>
<tr>
<td>3.</td>
<td>&lt;</td>
<td>2</td>
<td>Mic/Op 0 11 1 0 0 Nor 14</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>7</td>
<td>Total 2 15 1 0 25</td>
</tr>
</tbody>
</table>

25 overt coat color chimeras were visually divided into 3 groups; (1) Elo (agouti) dominant (>), (2) balanced (=) and (3) normal (albino) dominant (<) groups. Mic, the lens of Elo-type with ruptured lens capsule; Op, opaque lens; Nor, the normal-sized clear lens.

*Two chimeras had a Elo-type lens in one side and a opaque lens in another side.

**One chimera had a normal-sized clear lens and a opaque lens.

Fig. 5. Three different types of lenses from Elo/+↔+/+ chimeric mice at 30 days. (A) Small and deformed lens rudiment (Elo-type). (B) Opaque lens. (C) Normal-sized, clear lens. Bar: 2 mm.

morphology, which were not detected in this region of the Elo lens, were present together with the Elo-type nuclei. Figs 6C and D show a chimeric lens and normal control from day 18 embryos. In the cortex of the chimera's lens, both morphologically normal and abnormal nuclei were present (Fig. 6C). The lens capsule was completely formed in the posterior region. The morphological features of the abnormal nuclei were similar to those seen in the Elo lens, i.e. condensed nuclear chromatin marginated to nuclear membrane. These Elo-type nuclei remained in the posterior region of the lens, while normal nuclei were regularly arranged in the anterior region (Fig. 6D).

Immunohistochemical analysis of chimerism in embryonic eye tissue with anti-CSA antibody

To determine the strain origin of the deficient lens fiber cells in chimeras, tissue sections of chimeric embryos were immunohistochemically stained with anti-CSA antibody. Six chimeric embryos at day 14.5 were observed, and the proportion of both genotypes varied among individual chimeras. The proportion of each genotype in chimeric lens did not correspond to that in the neural retina in each chimeric mouse. Fig. 7A–E shows CSA-stained sections of the eye tissue from different individuals. In Fig. 7A, CSA-negative cells (+/+ ) are predominant over positive cells in both neural retina and lens. The CSA-positive cells were detected in the lens epithelium, elongating fiber cells in the lens cortex, and globular cells in the central region (Fig. 7B). Note that the CSA-positive globular cells have abnormal nuclei which are characteristic of the Elo lens. In the central region, the fiber cell arrangement of the lens was disorganized in spite of the presence of CSA-negative cells. Fig. 7C shows a +/+ -dominant lens developed with Elo/+ -dominant neural retina. In this lens, a few CSA-positive fiber cells with abnormal nuclei were detected together with negative cells with normal nuclei (Fig. 7D). Of interest, the positive cells did not seem as globular as those observed in the Elo lens. The negative cells were fully elongated. When CSA-positive cells predominate in both lens and retina, the lens structure is similar to the mutant lens (Fig. 7E). These observations indicate that the degree of disorganization of the fiber structure is correlated to the proportion of CSA-positive cells in the fiber area, and that the cells with morphologically abnormal nuclei derived only from C3H-Elo/+ , not from BALB/c-++/+.

Discussion

As a result of the chimeric experiment, the rupture of the lens capsule was rescued in some normal cell dominant chimeras. Immunohistochemical analysis of chimerism revealed that the proportion of both genotypes varied in the chimera's lenses. In the Elo/+ dominant lens, Elo/+ fiber cells became globular, and their nuclei were morphologically abnormal and local-
Fig. 6. Longitudinal sections of lenses from Elo/++/+ chimeric mice. (A) The Elo-type lens. The lens capsule is ruptured at the posterior region, and the lens cells are seen outside of the original lens area. (B) The opaque lens. Note pigmented epithelium of iris is chimeric (A and B, arrows). (C) Lens of chimera at day 18 embryo. Elo-type nuclei of lens fiber cells are seen in the posterior region of the lens (arrows). Pigmented (p) and non-pigmented areas in the retinal pigment epithelium indicate that this embryo is chimeric. (D) Lens of normal control (BALB/cA) at day 18. No abnormal nuclei are detected. Bars: (A,B) 200 \mu m, (C,D) 100 \mu m. Sections were stained with HE.

ized in posterior region. The fiber arrangement of +/- cells around a few Elo/+ cell groups was disturbed (Fig. 7A, B). These lenses may develop to be opaque or Elo-type lenses in adulthood (Fig. 5A, B). It can be surmised that even normal lens fiber cells may be affected in their elongating process by surrounding mutant cells. While, in the +/- dominant lens, Elo/+ fiber cells had also morphologically abnormal nuclei, which were located in posterior lens region (Fig. 7C, D). However, these Elo/+ cells were not globular in shape as in the Elo lens. The fiber structure around the Elo/+ cells seemed to be well-organized. The morphological abnormality in the Elo nucleus appears to be independent from the globular cell formation. It is likely that the normal-sized clear lens observed in one adult chimera (Table 5) may contain a few Elo/+ derived cells which did not have globular cytoplasm. From our chimera studies, both mutant and normal fiber cells seem to communicate with each other closely, and they must affect the elongating process either positively or negatively during lens development. The cell–cell interactions in the fiber cell population must be very important for fiber cell elongation, but does not affect the nuclear morphology.

Chronological observations indicate that the Elo gene primarily affects the differentiation of lens fiber cells. As revealed by the quantitative analysis of the developing lens epithelium (Tables 1–3 and Fig. 4), the Elo lens epithelium had significantly smaller numbers of cells than the normal one. While the number of the S-phase cells significantly decreased with aging in both genotypes, these cell numbers appeared not to be affected by Elo gene. However, the analysis of variance in the incidence of S-phase cells during lens development made it clear that Elo gene definitely influenced the incidence of S-phase cells with aging. As far as we analyzed, there was no obvious evidence that Elo gene is positively influencing the cell proliferation of lens epithelium. Here, we would speculate that reduction of a total cell number in the Elo lens epithelium may heavily depend upon malformation due to lens fiber cell deficiency in their morphology and that the cell in Elo lens epithelium may displace away from the epithelium region much faster than the cells in the normal.
Fig. 7. Immunohistological staining of the chimerism with anti-CSA antibody in the lens and retina of C3H-Elo/+→ BALB/c chimeras at day 14.5 embryo. Sections of different embryos (A; B, C; D, E). DAB reaction products were intensified by DAB/gold/silver amplification system. Cells derived from the C3H-Elo/+ are CSA-positive, and are visualized by deposition of black silver grains in the cell cytoplasm. Sections were counterstained with HE. (A) CSA-positive lens cells are distributed to the lens epithelium, elongating fibers and central region of the lens. In the neural retina, positive cells are noted as several radial patches (arrows). (B) High magnification of A. In the deep cortex and center of the lens, lens cells with abnormal nuclei are CSA-positive (arrowheads), but cells with normal nuclei are negative. In the peripheral cortex of the lens, CSA-positive elongating cells are detected (arrow). Note the fiber structure in the center of the lens is disturbed. (C) Neural retina is dominated by CSA-positive cells. In contrast, only a few positive cells are seen in the lens. (D) Positive cells (arrows) in the central fiber area have morphologically abnormal nuclei located inferior to the normal nuclei (n). (E) In the lens and retina, positive cells are predominant. The lens is morphologically similar to the Elo. Bars: (A,C,E) 200 μm, (B,D) 50 μm.

Consequently, the epithelium in the Elo lens seems to cover a much smaller area than the normal type epithelium. Immunohistological observations of BrdU incorporation revealed that the distribution patterns of BrdU-incorporated cells in the Elo lens epithelium was also different from that in the normal epithelium. We have also preliminarily analyzed the distribution of S-phase cells in each genotype. Briefly, as shown in Fig. 8, the histological part of the lens epithelium was divided into 4 regions. In normal lens epithelium, approximately 40% of S-phase cells locate in the region I. The cell numbers gradually decreased toward region IV. This tendency was very steady through the whole developmental stages. While, in Elo lens, until day 13 of gestation, this pattern was similar to normal lens, but after day 14, this peak shifted to the region II. At day
18, S-phase cells almost evenly distributed in the regions I–III. These different patterns of S-phase cell distribution may be also caused by the lens malformation due to morphological deficiency in the lens fiber cells and to rupture of the lens capsule. As the lens fiber differentiation is morphologically defined by cell elongation, the Elo gene appears to affect lens fiber cell differentiation rather than lens cell proliferation, and to be expressed cell autonomously in the differentiating fiber cells.

It is well known that the following factors may be involved in the lens development: (1) lens-cell-specific proteins, crystallins, especially α-, β- and γ-crystallins (McAvoy, 1978a), (2) retinal factors that can induce the lens epithelial cell elongation (Muthukkaruppan, 1965) and (3) the lens capsule, which may be important for the lens differentiation (Hendrix and Zwaan, 1974). The differentiation of lens fiber cells is characterized by the synthesis of lens-specific proteins, such as γ-crystallins (Maurer-Orlando et al. 1987). Two-dimensional gel electrophoresis studies indicated that γ2-crystallin is substantially reduced in the mutant lens (Masaki and Watanabe, personal communication). The Elo gene is known to be closely linked to the γ-crystallin genes (Skow 1982), but it is apparently not a γ-crystallin structural gene (Quinlan et al. 1987). Quinlan et al. (1987) also reported that synthesis of γ-crystallins in the mutant lens is reduced at transcriptional level. We observed eosinophilic granules in normal lens from day 14 embryos. Their distribution was restricted in the lens fiber cells except for those in the lens cortex. However, these granules were not detectable in the lens fiber cells of the Elo lens. We have immunohistochemically observed the expression pattern of α-, β- and γ-crystallin proteins during embryonic lens development in the mutant, but were unsuccessful in detecting any significant difference from the normal (data not shown). However, since the distribution of γ-crystallin and that of eosinophilic granules overlapped well, eosinophilic granules may contain γ-crystallin proteins, but we lack direct evidence. Furthermore, the lens fiber elongation is supported by the lens cytoskeleton, such as actin, tubulin and vimentin (Sax et al. 1990). These facts lead us to suggest that the Elo gene may produce an intracellular inhibitory factor which affects the transcription of γ-crystallins or cytoskeletal organization. It is also reported that the neural retina has an inductive influence on fiber cell elongation (Coulombre and Coulombre, 1963). The existence of retinal factors has been shown in the development of the mouse lens in vitro (Muthukkaruppan, 1965). Recently, fibroblast growth factors from neural retina were identified as potent fiber-cell-inducing molecules (McAvoy and Chamberlain, 1989). The previous study of organ culture of the Elo embryonic eye tissue has shown that pathogenesis of the Elo is independent of the extracellular environment (Watanabe et al. 1980). However, lens–retinal interactions in the Elo have not been investigated yet. In this study, we have demonstrated that the exclusively Elo/+–dominant neural retina can support full elongation of +/+ lens fiber cells (Fig. 7C). Therefore, possibilities of deficiency in retinal factors should be ruled out in this mutant. In the Elo lens, large globular cells were observed in the center of the lens, and the lens capsule was ruptured in the posterior region. When the capsule of an early embryonic lens was removed, elongated lens fiber cells rounded up into large spherical cells (Muggleton-Harris et al. 1981). Fragments of lens capsule can promote cloned mouse lens epithelial cells to differentiate into a lentoid body in culture (Muggleton-Harris and Higbee, 1987). Their results suggested that the capsule may regulate lens cell differentiation. However, it is unlikely that, in the Elo lens, the mutant gene primarily affected the synthesis of lens capsule materials, because in the opaque lens of the chimeras, the mutant phenotype of lens cells was observed in spite of complete formation of the lens capsule (Fig. 6C). Analysis of embryonic chicken lens development reported that lens volume expansion has occurred predominantly in the posterior region (Linsenmayer et al. 1984). Lens capsule materials were synthesized and secreted by both epithelial and fiber cells (Rafferty and Goosens, 1978). Then, defects in the capsular materials in the early development may result in the rupture of the posterior region in later embryonic stage. In those opaque lenses of chimeras, the normal lens fibers may secrete sufficient capsular materials to sustain the rapid growth of the lens volume. Thus, the rupture of the lens capsule in the Elo lens may be a secondary effect of the mutant gene.

Finally, we would conclude that the Elo gene of the mouse may primarily affect the differentiation of lens fiber cell, resulting in reduced elongation and abnormal nuclear morphology. The secondary defects such as rupture in the lens capsule could be rescued in some chimeras because the capsule materials secreted extra-cellularly by normal cells can provide much improved cellular environment for the lens development. From chimera study, we conclude that interactions between developing lens fiber cells should be studied to determine the cellular or molecular mechanism of fiber cell elongation. The Elo mouse is particularly suited for future studies on the regulatory mechanism of lens fiber differentiation, including identification of the mutant gene and its normal counterpart.
References

BURNS, J., CHAN, V., JONASSAN, J., FLEMING, K., TAYLOR, S. AND
biotinylated DNA probes hybridised in situ: rapid sex

development: Fiber elongation and lens orientation. Science 224,
1489–1490.

GREEN, M. C. (1981). Catalog of mutant genes and polymorphic
loci. In Genetic Variants and Strains of the Laboratory Mouse

HANAZONO, M., YOSHII, A., OTA, K., KITO, J. AND KUSAKABE,
M. (1990). Immunohistochemical detection of DNA replication
in mouse uterine cells by bromodeoxyuridine labeling of wax-
and resin-embedded tissue sections. Stain Technol. 65, 139–149.

HANNA, C. AND O'BRIEN, J. E. (1961). Cell production and
migration in the epithelial layer of the lens. Archs Ophthal. 66,
103–107.

glycoprotein concentration of the extracellular matrix between
lens and optic vesicle associated with early lens differentiation.
Differentiation 2, 357–362.

HOGAN, B., CONSTANTINI, F. AND LACY, E. (1986). In Manipulating
the Mouse Embryo. Cold Spring Harbor Laboratory, U.S.A.

Biochemistry of basement membranes. Int. Rev. Cytol. 61,
167–228.

KONYUKH, B. V. (1987). Gene expression and cell interactions
Biol. 1, 561–601.

KUSAKABE, M., SAKAKURA, T., NISHIZUKA, Y., SANO, M. AND
technique for immunohistochmistry. Stain Technol. 59,
127–132.

KUSAKABE, M., YOKOYAMA, M., SAKAKURA, T., NOMURA, T.,
for analysis of cell distribution in chimeric mouse organs using a

Basement membrane structure and assembly: Influences from
immunological studies with monoclonal antibodies. In The Role
of the Extracellular Matrix in Development (ed. Trelstad R. L.),

MAUER-ORLANDO, M., PATTERSON, R. C., LOK, S., TSUL, L.-C.
AND BREITMAN, M. L. (1987). Differential regulation of γ-
crystallin genes during mouse lens development. Devl Biol. 119,
260–267.

MCAVOY, J. W. (1978a). Cell division, cell elongation and
distribution of α, β and γ-crystallins in the rat lens. J. Embryol.

MCAVOY, J. W. (1978b). Cell division, cell elongation and the co-
ordination of crystallin gene expression during lens

Fibroblast growth factor (FGF) induces different responses in lens
epithelial cells depending on its concentration. Development 107,
221–228.

MINTZ, B. (1971). Allophenic mice of multiembryo origin. In

Phytohemagglutinin mediated blastomere aggregation and
development of allophenic mice. Devl Biol. 31, 155–199.

Rescue of developmental lens abnormalities in chimaeras of non-catarractous and congenital cataractous mice. Development 99,
473–480.

modulating mouse lens epithelial cell morphology with
differentiation and development of a lentoid structure in vitro.
Development 99, 25–32.

In vitro characteristics of normal and cataractous mouse lens

MUTHUKARUPPAN, V. (1965). Inductive tissue interaction in the
development of the mouse lens in vitro. J. exp. Zool. 159,
269–286.

ODA, S., WATANABE, K., FUJISAWA, H. AND KAMEYAMA, Y.
(1980b). Impaired development of lens fibers in genetic
microphthalmia, eye lens obsolescence, Elo, of the mouse. Expl
Eye Res. 31, 673–681.

ODA, S., WATANABE, T. AND KONDO, K. (1980a). New mutation,
eye lens obsolescence, Elo on chromosome 1 in the mouse.
Japan J. Genetics 55, 71–75.

The mouse eye lens obsolescence (Elo) mutant: Studies on
crystallin gene expression and linkage analysis between the


SAX, C. M., FARKKEL, F. X., ZEHNER, Z. E. AND PIATIGORSKY, J.
(1984). Regulation of vimentin gene expression in the ocular

SCHUTTE, B., REYNDS, M. J. M., BOUSMAN, F. J. AND BLIJHAM,
G. H. (1987). Effect of tissue fixation on anti-
bromodeoxyuridine immunohistochmistry. J. Histochem.
Cytochem. 35, 1343–1345.

variation in mouse γ-crystallins. Expl Eye Res. 34, 509–516.

WATANABE, K., FUJISAWA, H., ODA, S. AND KAMEYAMA, Y.
(1980). Organ culture and immunohistochemistry of the genetically
malformed lenses, in eye lens obsolescence, Elo, of the mouse.
Expl Eye Res. 31, 683–689.

(Accepted 11 September 1991)