

## Disruption of *Gja8* ( $\alpha 8$ connexin) in mice leads to microphthalmia associated with retardation of lens growth and lens fiber maturation

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### SUMMARY

The development of the vertebrate lens utilizes a sophisticated cell-cell communication network via gap junction channels, which are made up of at least three connexin isoforms,  $\alpha 8$ Cx50),  $\alpha 3$  (Cx46) and  $\alpha 1$  (Cx43), and which are encoded by three different genes. In a previous study, we reported that, with a disruption of *Gja3* ( $\alpha 3$  connexin), mice developed nuclear cataracts with a normal sized lens. We show that *Gja8<sup>tm1</sup>* ( $\alpha 8$ -/-) mice develop microphthalmia with small lenses and nuclear cataracts, while the  $\alpha 8$  heterozygous (+/-) mice have relatively normal eyes and lenses. A comparative study of these  $\alpha 3$  and  $\alpha 8$  knockout mice showed that the protein levels of both  $\alpha 3$  and  $\alpha 8$  were independently regulated and there was no compensation for either the  $\alpha 3$  or  $\alpha 8$  protein from the wild-

type allele when the other allele was disrupted. More interestingly, western blotting data indicated that the presence of  $\alpha 8$  in the lens nucleus is dependent on  $\alpha 3$  connexin, but not vice versa. The staining of the knock-in *lacZ* reporter gene showed the promoter activity of  $\alpha 8$  connexin is much higher than that of  $\alpha 3$  connexin in embryonic lenses and in adult lens epithelium. More importantly, a delayed denucleation process was observed in the interior fibers of the  $\alpha 8$ -/- lenses. Therefore,  $\alpha 8$  connexin is required for proper fiber cell maturation and control of lens size.

Key words: Connexin, Lens, Microphthalmia, Cataract, Mouse

### INTRODUCTION

The ocular lens provides us with one of the simplest systems for studying several fundamental biological processes, including cell proliferation, differentiation, and maturation (Wride, 1996; Piatigorsky, 1981). The lens is composed of a monolayer of cuboidal epithelial cells covering the anterior surface of a buck of elongated fibers. All lens cells are wrapped in a collagen-based capsule. During early embryonic development, the monolayer of the ectoderm adjacent to the optic vesicle undergoes a sequential process of thickening, invaginating and pinching off, eventually forming the lens vesicle. The posterior cells of the lens vesicle then begin to differentiate and elongate, forming the primary lens fibers, while the anterior cells of the lens vesicle remain as a monolayer epithelium. The epithelial cells near the lens equator continue to proliferate, and the grown epithelial cells that pass the equatorial line subsequently differentiate into secondary fiber cells that lie on the top of the primary fibers. The growth of the lens is dependent upon the production of secondary fiber cells from the anterior epithelial cells throughout the life span of the organism.

The differentiating fibers eventually lose all their intracellular organelles, such as the nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, etc., to become

mature fibers in the deep cortical region of the lens (Bassnett and Beebe, 1992). This maturation of the fibers is one of the critical properties of the lens in its function of transmitting a light image onto the retina. If there are intracellular organelles in the interior region of the lens, this will cause light scattering, or so-called 'cataracts'. Because the interior mature fibers lose all their intracellular organelles, they have an extremely low metabolic activity and depend mainly on the epithelium and peripheral differential fibers for maintenance. Therefore, out of necessity, the lens has developed a sophisticated cell-cell communication network that includes gap junction channels, which facilitate both an active metabolism and the transport of small metabolites, such as ions, water and secondary messengers, in order to maintain its transparency (Goodenough, 1992; Mathias et al., 1997).

The development and function of the vertebrate lens utilizes gap junction channels that are formed by the products of at least three connexin genes:  $\alpha 1$  (Cx43),  $\alpha 3$  (Cx46) and  $\alpha 8$  (Cx50) (Beyer et al., 1987; Kistler et al., 1988; Paul et al., 1991; White et al., 1992). Connexin  $\alpha 1$  is restrictively expressed in the lens epithelial cells, and its protein can also be detected in the newly differentiating cells in the bow region of the lens. The lens fibers mainly utilize  $\alpha 3$  and  $\alpha 8$  connexins, which are two connexin isoforms colocalized in the same gap junction plaque in the plasma membrane of fiber cells (Gong

et al., 1997; Benedetti et al., 2000).  $\alpha 8$  connexin has also been detected in lens epithelial cells (Dahm et al., 1999), and this suggests that different regulatory machinery may control the expression of  $\alpha 3$  and  $\alpha 8$  connexin genes in lens epithelial cells, despite the fact that the same factors may control their expression in fiber cells.

In one of our previous studies, we reported that *Gja3<sup>tml</sup>* ( $\alpha 3^{-/-}$ ) mutant mice developed nuclear cataracts to varying degrees, depending on the genetic background of the strain (Gong et al., 1997; Gong et al., 1999). In order to compare the functional roles of  $\alpha 3$  and  $\alpha 8$  connexin in vivo, in this study we have generated  $\alpha 8$  knockout mice with a knock-in *lacZ* reporter gene, similar to that in our previous studies on  $\alpha 3$  knockout mice with a knock-in *lacZ* reporter gene (Gong et al., 1997). In this way, we were able to: (1) compare the phenotypic changes between  $\alpha 8$  and  $\alpha 3$  knockout mice; (2) examine the expression pattern of the knock-in *lacZ* reporter gene during lens development in the two knockout mice in parallel; and (3) try to find distinctive morphological and biochemical alterations in the two knockout mice. This study provides some important insights into the utilization of both  $\alpha 8$  and  $\alpha 3$  connexins in the lens.

## MATERIALS AND METHODS

### Generation of the $\alpha 8$ knockout mice

A 16 kb genomic DNA fragment containing the coding exon for  $\alpha 8$  connexin was isolated from a 129/Sv FIX mouse genomic library (Stratagene) that was screened using standard procedures with an  $\alpha 8$  cDNA probe. This genomic DNA fragment (a part of its physical map is shown in Fig. 1), was used to construct an  $\alpha 8$  gene targeting vector with a knock-in *lacZ* reporter gene. In order to create  $\alpha 8$  knockout with an in-frame knock-in *lacZ* reporter gene (Bonnerot et al., 1987) that was under the control of the endogenous  $\alpha 8$  gene promoter, a 200 bp PCR fragment (*Bam*HI-*Sma*I) was generated to create a novel *Sma*I site in the 6<sup>th</sup> codon of  $\alpha 8$  connexin gene. DNA sequence of the PCR fragment was confirmed to be identical to the original sequence of the  $\alpha 8$  genomic fragment except for the *Sma*I site. Using the 200 bp PCR fragment (*Bam*HI-*Sma*I) as a linker to connect an upstream 4 kb  $\alpha 8$  gene fragment (*Not*I-*Bam*HI) and a downstream *lacZ* reporter gene (*Sma*I-*Xho*I), a 4.2 kb 5' homologous arm of  $\alpha 8$  connexin gene following an in-frame knock-in *lacZ* reporter gene (*Not*I-*Xho*I) was constructed, then inserted into the *Not*I-*Xho*I sites upstream of the *pgkNeo* gene in the pPNT vector (Tybulewicz et al., 1991). Subsequently, 1.4 kb of the 3'  $\alpha 8$  genomic fragment (*Pst*I-*Xba*I), including a small 3' portion of the exon 2, was ligated in the downstream of *pgkNeo*. The genomic sequence encoding all 4 transmembrane domains of the  $\alpha 8$  connexin was deleted in the  $\alpha 8$  gene targeting vector. The targeting vector was linearized at a unique 5' *Not*I site, then electroporated into R1 ES cells. G418-resistant clones were isolated and screened for a disrupted  $\alpha 8$  gene allele by a 3' external DNA probe, in order to detect a 7.5 kb *Bam*HI RFLP (Fig. 1). Knockout clones were identified and used for generating chimeras by injecting knockout ES cells into C57BL/6J (B6) blastocysts. The male chimeras were mated with B6 females to produce the F<sub>1</sub>  $\alpha 8$  heterozygous (+/-) knockout mice. The  $\alpha 8$  homozygous (-/-) knockout mice were generated from an intercross between F<sub>1</sub>  $\alpha 8$ +/- knockout mice.

PCR was used to genotype the wild-type and knockout mice. A 320 bp PCR fragment was expected from the wild-type allele of *Gja8*( $\alpha 8$ ) by using a pair of primers: sense GGATCCTTTCAAACAAC and anti-sense GCCGATGACAGTGGAGTGCTC; and a 450 bp PCR fragment from the targeted mutant allele of *Gja8* using a pair

of primers: sense GGATCCTTTCAAACAAC and anti-sense CAGGGTTTTCCCAGTCACGAC.

### Histological, immunohistochemical and *lacZ* expression analysis

Standard histological methods were used for analyzing different mutant mice (Lovicu and Overbeek, 1998). The  $\beta$ -galactosidase staining method was carried out as previously described (Bonnerot and Nicholas, 1993).

Frozen sections of lenses were prepared and used for the antibody staining, following the procedure described in our previous paper (Gong et al., 1997). We used a mouse monoclonal antibody (6-4-B2-C6, provided by Dr Kistler) (Bond et al., 1996) for  $\alpha 8$  connexin and a rabbit polyclonal antibody against the intracellular loop region of the  $\alpha 3$  connexin, the same antibody used in a previous study (Gong et al., 1997) for double immunolabeling. A deconvolution light microscope (Delta Vision Optical Sectioning Microscope Model 283) was used to examine the fluorescence of the indirect immunostaining.

### Electron microscopic analysis

For freeze-fracture electron microscopic analysis, the lenses were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 5 days, then processed and analyzed as described previously (Benedetti et al., 2000; Risek et al., 1994). For transmission electron microscopic analysis, the lenses were postfixed in 1% OsO<sub>4</sub>, stained with 0.5% tannic acid/0.05 M cacodylate buffer, and neutralized with 1% Na<sub>2</sub>SO<sub>4</sub> in 0.1 M cacodylate buffer. The lenses were then stained en bloc with 1% uranyl acetate/10% ethanol, and further dehydrated in a standard ethanol series (the ethanol was exchanged with HPMA). Thereafter, the lenses were infiltrated overnight in a 1:1 HPMA/LX112 (Ladd Scientific) mixture while rotating, immersed in 100% LX112 for 4 hours, then embedded in LX112. They were polymerized for 24-36 hours at 60°C. A standard method was used for thin sectioning and they were examined with a Philips CM100 electron microscope.

### Western blotting

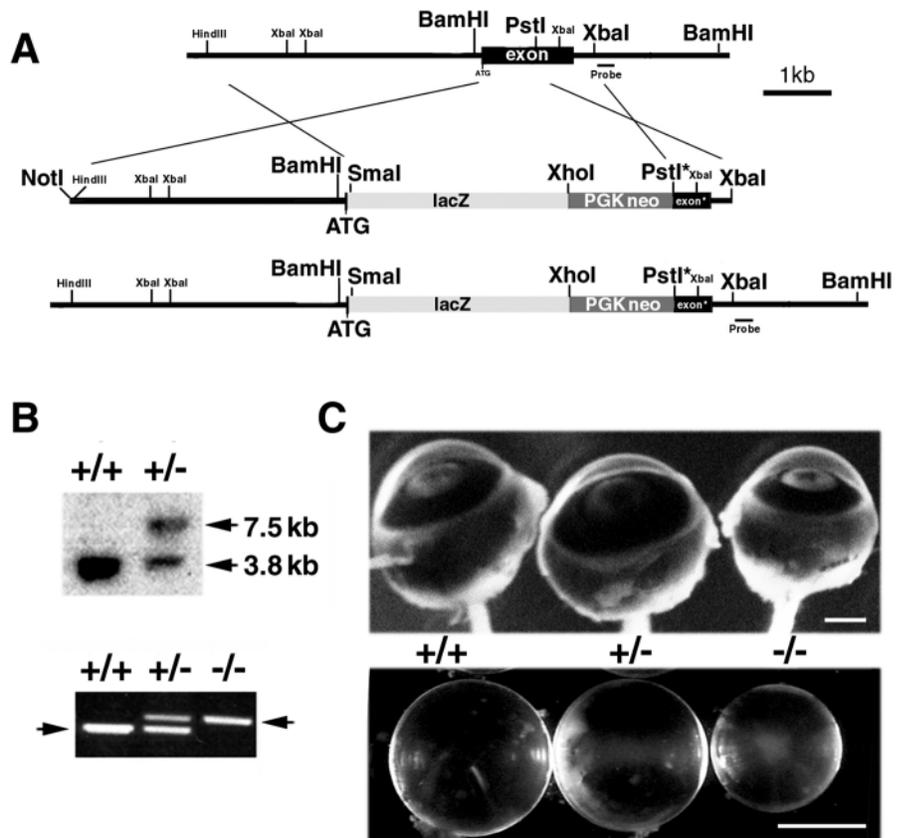
Different lens homogenates were prepared as described previously (Gong et al., 1997; Gong et al., 1999). For quantitative western blotting, the lens proteins were dissolved in lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerolphosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1  $\mu$ g/ml leupeptin, 1 mM PMSF), and the protein concentration was measured using a MicroBCA protein assay kit from Pierce Chemicals. Equal amounts of these lens protein samples were loaded and run onto SDS-PAGE, then proteins were transferred onto nitrocellulose membranes and detected using specific antibodies with standard protocols. Rabbit polyclonal antibodies against  $\alpha 8$  connexin (generously provided by Dr Thomas White),  $\alpha 3$  connexin and MP26 were used for western blotting.

## RESULTS

### Development of microphthalmia with a small lens in the connexin $\alpha 8^{-/-}$ mice

Fig. 1A shows physical maps of the  $\alpha 8$  targeting vector, a wild-type allele, and the disrupted mutant allele of the  $\alpha 8$  connexin gene. A *lacZ* reporter gene was inserted in-frame downstream of the 6<sup>th</sup> codon of the  $\alpha 8$  gene. The R1 embryonic clones with disrupted  $\alpha 8$  genes were identified by Southern blot analysis (Fig. 1B, upper panel). Both homozygous  $\alpha 8^{-/-}$  and heterozygous  $\alpha 8$ +/- knockout mice were viable and fertile. The genotypes of the knockout mice were verified by Southern blotting or PCR (Fig. 1B, bottom panel). All comparison

**Fig. 1.** Homozygous  $\alpha 8$  knockout mice developed microphthalmia with mild nuclear cataracts. (A) Physical maps of the wild-type *Gja8* allele, the  $\alpha 8$  targeting vector, and the disrupted *Gja8* allele. The DNA probe used for Southern blotting is indicated on the corresponding region of the wild-type and knockout *Gja8* alleles. A 7.5 kb band corresponded to the knockout allele, and a 3.8 kb band corresponded to the wild-type allele. (B) The upper panel shows a blot of an embryonic stem cell clone with a disrupted *Gja8* allele (+/-), in comparison with the wild-type control (+/+). This was verified by Southern blotting using a DNA probe, labeled in A. The lower panel shows the PCR results from homozygous  $\alpha 8$  knockout (-/-), heterozygous  $\alpha 8$  knockout (+/-) and wild-type (+/+) mice. The left arrow indicates a 320 bp band from the wild-type allele and the right arrow for a 450 bp band from the knockout allele. (C) Phenotypic comparison of the eyes and lenses from homozygous  $\alpha 8$  knockout (-/-), heterozygous  $\alpha 8$  knockout (+/-), and wild-type (+/+) sibs. Scale bar, 1 mm.



studies were carried out on the siblings of these knockout mice.

The  $\alpha 8$ +/- mice appeared to have normal eyes and lenses, while the  $\alpha 8$ -/- mice developed microphthalmia with smaller lenses (Fig. 1C). The size and weight of the lenses of the adult  $\alpha 8$ -/- mice were around 60% that of the lenses of their wild-type (+/+) littermates ( $n=20$ ). Very mild nuclear cataracts were observed in the  $\alpha 8$ -/- lenses of the adult mice (Fig. 1C).

No  $\alpha 8$  connexin proteins were detected in the lens homogenates of adult  $\alpha 8$ -/- mice, while half the amount of  $\alpha 8$  connexin proteins, as determined by densitometric analysis ( $n>3$ ), was detected in the lens homogenates of the  $\alpha 8$ +/- sibs, when compared to their wild-type counterparts (Fig. 2). According to western blot analysis, there were no obvious changes in the  $\alpha 3$  connexin or the major lens membrane protein (MP26 or aquaporin-0) in the lens homogenates of  $\alpha 8$ -/- mice, as compared to the  $\alpha 8$ +/+ and  $\alpha 8$ +/- mice (Fig. 2). Moreover, no additional changes in the  $\alpha$ -,  $\beta$ -, or  $\gamma$ -crystallins were detected in the 3-week-old lenses using their specific antibodies (data not shown), aside from a reduction in the amount of total crystallins due to the small size of the  $\alpha 8$ -/- lenses. Substantial degradation of the crystallins such as the cleavage of  $\gamma$ -crystallin in the  $\alpha 3$ -/- lenses (Gong et al., 1997) was not found in the  $\alpha 8$ -/- lenses.

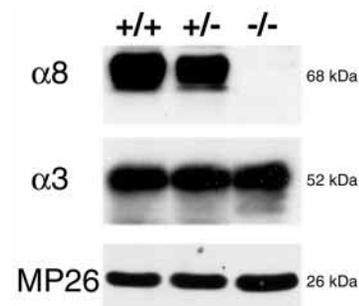
#### A much smaller gap junction plaque observed in the $\alpha 8$ -/- lens fibers

Although the  $\alpha 8$ -/- mice developed much smaller lenses, no obvious morphological changes in the lens epithelial cells or fibers were found in standard histological sections (Fig. 3A).

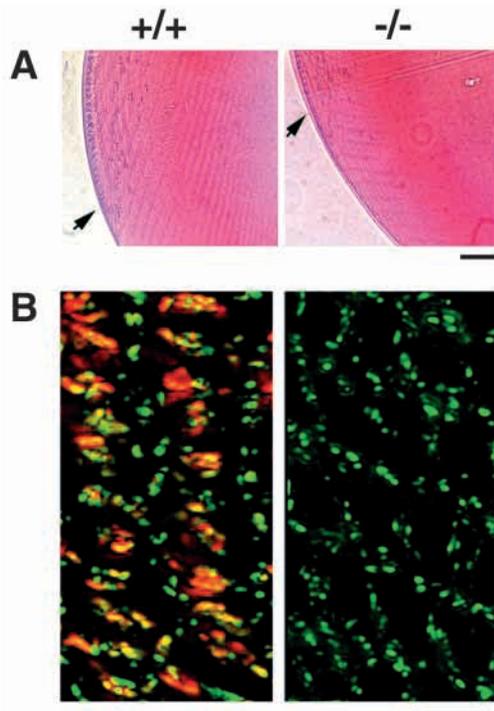
According to double immunolabeling results obtain from laser confocal or light microscopy, we among others published that  $\alpha 8$  and  $\alpha 3$  connexins are mainly colocalized in the same fluorescent spots in the plasma membrane (assuming equivalency to the gap junction plaques observed by freeze-

fracture EM) (Gong et al., 1997). However, through the use of an improved light microscopy system with a computational deconvolution data process, we have found that  $\alpha 8$  (red) and  $\alpha 3$  (green) connexins were actually segregated from each other in the same fluorescent spot in the plasma membrane of lens fibers (Fig. 3B, left panel) with a small amount of colocalization.

Consistent with western blot results, no  $\alpha 8$  protein was detected in the frozen lens sections from the  $\alpha 8$ -/- mice by a double immunolabeling for  $\alpha 3$  and  $\alpha 8$ , using their specific antibodies (Fig. 3B, right panel). Interestingly, the fluorescent spots for  $\alpha 3$  connexin, detected in the  $\alpha 8$ -/- sections, were



**Fig. 2.** Western blots from the lens homogenates of homozygous  $\alpha 8$  knockout (-/-), heterozygous  $\alpha 8$  knockout (+/-), and wild-type (+/+) littermates. Equal amounts of the lens homogenates (50  $\mu$ g per lane) were loaded and separated by SDS-PAGE, then detected by polyclonal antibodies against  $\alpha 8$  connexin,  $\alpha 3$  connexin, and MP26. Molecular masses are listed on the right-hand side.



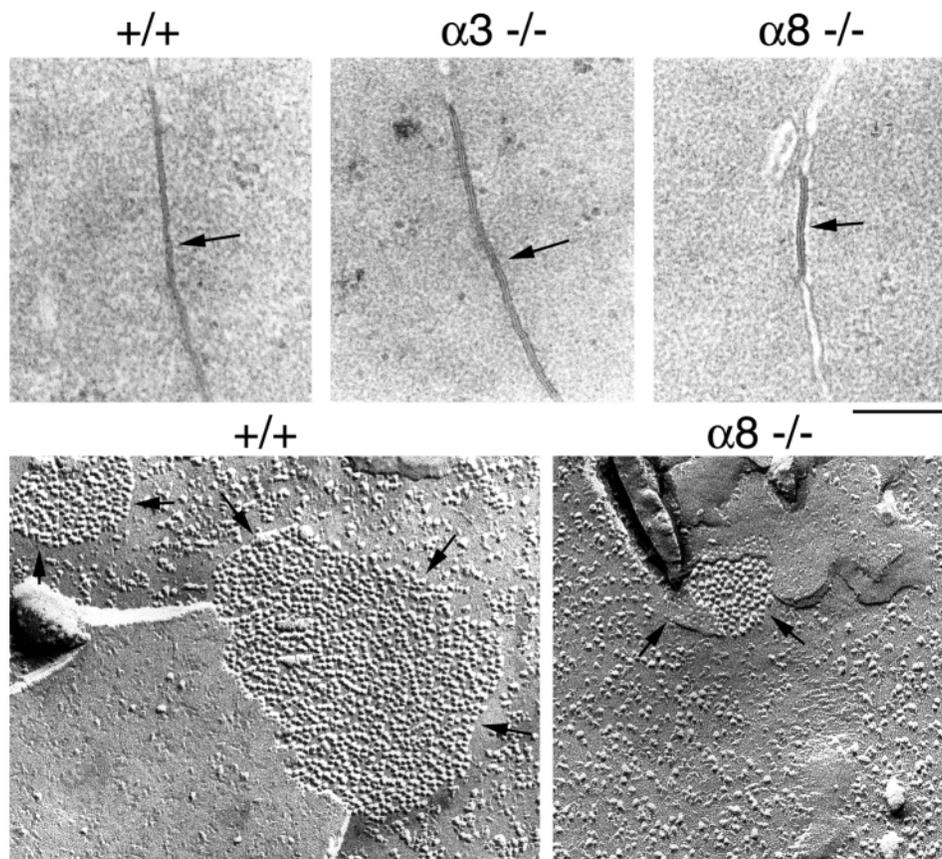
similar in size to the small  $\alpha 3$  connexin spots (green) in wild-type lens sections (Fig. 3B, left panel), but there was additional  $\alpha 3$  connexin (orange) which was colocalized with the  $\alpha 8$  connexin to form larger fluorescent spots.

Gap junctions in the  $\alpha 8^{-/-}$  lenses were further examined by both thin-section and freeze-fracture electron microscopy (Fig. 4). A much smaller gap junction was observed in the cortical fibers of the  $\alpha 8^{-/-}$  lenses than in those of wild-type lenses by EM. The largest gap junction that we observed from more than eight  $\alpha 8^{-/-}$  samples was around 60 nm in length while most of gap junctions in wild-type or  $\alpha 3^{-/-}$  lenses were 4 times longer (Gong et al., 1997).

#### Presence of $\alpha 8$ connexin in the lens nucleus is dependent on the existence of $\alpha 3$ , while $\alpha 3$ connexin is independent of $\alpha 8$

The maturation of lens fibers is associated with a substantial proteolysis of both  $\alpha 3$  and  $\alpha 8$  connexins. We have further

**Fig. 4.** Gap junctions are smaller in  $\alpha 8^{-/-}$  lenses. Gap junctions in the lens cortical fibers from wild-type (+/+),  $\alpha 3^{-/-}$  (-/-), and  $\alpha 8^{-/-}$  mice were examined by thin-section (upper panels) and freeze-fracture (lower panels) electron microscopy. Gap junctions are indicated by arrows. Scale bar, 60 nm.

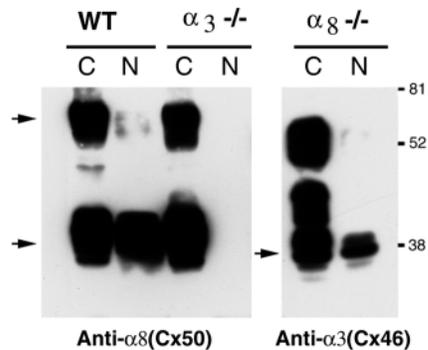


**Fig. 3.** (A) Representative histological sections from the bow region of wild-type (+/+) and  $\alpha 8$  homozygous knockout (-/-) lenses. The arrows indicate the lens bow region where epithelial cells differentiate to become fiber cells. Scale bar, 50  $\mu$ m. (B) Double immunolabeling of  $\alpha 8$  (red) and  $\alpha 3$  (green) connexins in the cortical fibers of the frozen sections from wild-type (+/+) and  $\alpha 8$  homozygous knockout (-/-) lenses. Scale bar, 5  $\mu$ m.

examined  $\alpha 3$  and  $\alpha 8$  connexin in the homogenates prepared from the lens cortex (40% of the total lens weight) and the lens nucleus (60% of the total lens weight) of  $\alpha 8^{-/-}$  and  $\alpha 3^{-/-}$  adult mice. Consistent with the literature, phosphorylated (50-65 kDa) and cleaved forms of  $\alpha 3$  and  $\alpha 8$  (32-38 kDa) were detected in the homogenates of the lens cortex from wild-type,  $\alpha 8^{-/-}$  and  $\alpha 3^{-/-}$  lenses (Fig. 5). Mainly, the cleaved forms of  $\alpha 3$  and  $\alpha 8$  were detected in the lens nucleus of wild-type mice. To our surprise, not one of the cleaved  $\alpha 8$  connexin bands was detected in the homogenates of the  $\alpha 3^{-/-}$  lens nucleus (Fig. 5, the right-hand lane in the left panel), while the cleaved forms of  $\alpha 3$  were still found in the homogenates of the  $\alpha 8^{-/-}$  lens nucleus (Fig. 5, left panel).

#### The $\alpha 8$ -*lacZ* reporter gene expression was higher than that of the $\alpha 3$ -*lacZ* gene in embryonic lens cells and adult lens epithelial cells

The expression of knock-in *lacZ* gene activity was exclusively detected in the embryonic and adult lenses of  $\alpha 8$  knockout mice, and not in the other regions of the adult eye (Fig. 6A), which is similar to our previous findings about the knock-in *lacZ* reporter gene in  $\alpha 3$  knockout mice (Gong et al., 1997). We have further



**Fig. 5.** Western blots of lens cortical homogenates (C) and lens nuclear homogenates (N) from wild-type,  $\alpha 3$  homozygous knockout ( $-/-$ ), and  $\alpha 8$  homozygous knockout ( $-/-$ ) mice. The left panel shows the results obtained using an anti- $\alpha 8$  connexin antibody. The upper arrow indicates the intact  $\alpha 8$  connexin protein band, while the lower arrow points to the cleaved bands of  $\alpha 8$  connexin. The right panel shows the results obtained using an anti- $\alpha 3$  connexin antibody. The arrow indicates the cleaved forms of  $\alpha 3$  connexin. The molecular markers (in kDa) are listed on the right-hand side.

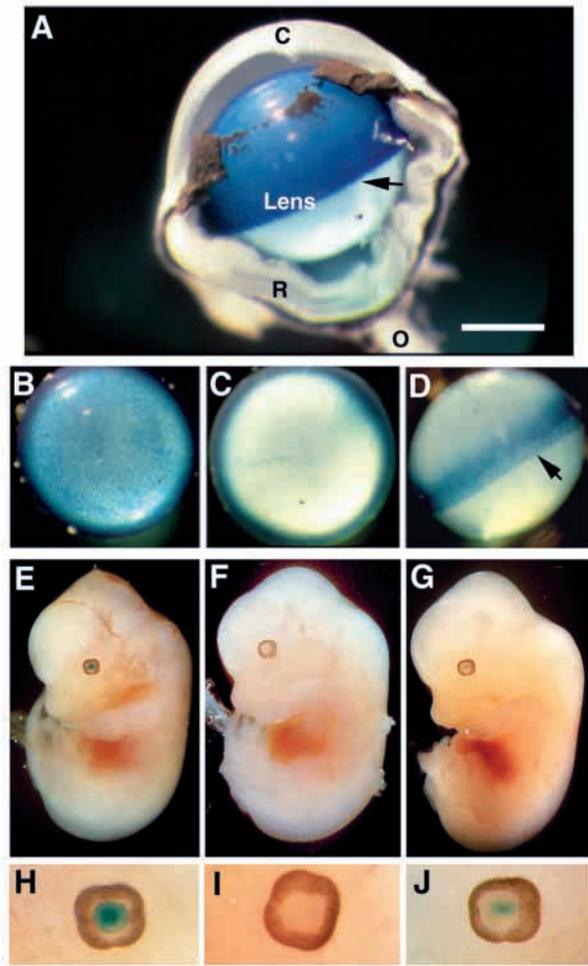
compared the knock-in *lacZ* activity between  $\alpha 8$  and  $\alpha 3$  knockout mice in the heterozygous knockout sibs, which were produced from an intercross between  $\alpha 8^{+/-}$  mice and  $\alpha 3^{+/-}$  mice (Fig. 6). The  $\alpha 8$ - $\beta$ -gal staining was much higher than the  $\alpha 3$ - $\beta$ -gal staining in the embryonic lens cells (Fig. 6H,J), and in the adult lens epithelium (Fig. 6B,C). Furthermore, the  $\alpha 8$ - $\beta$ -gal staining was uniformly high in all the lens epithelial cells, while the  $\alpha 3$ - $\beta$ -gal staining signal was relatively higher in the cells of the marginal zone, where proliferating epithelial cells are located (Fig. 6D), than the cells of the polar regions.

### The cellular nucleus of interior fibers was transiently sustained in the $\alpha 8^{-/-}$ lenses

The protein product of the knock-in *lacZ* reporter gene in the  $\alpha 8$  knockout mice (containing a nuclear-localization signal of the SV40 large T antigen) was mainly localized in the nuclei of lens epithelial cells and differentiating fiber cells. The  $\beta$ -gal staining in the cell nuclei of the interior fibers was normally eliminated during the fiber cell maturation process, so the mature interior fibers do not stain (Fig. 7C). Therefore, the posterior side of the  $\alpha 3$  and  $\alpha 8$  knockout lenses was also unstained (Fig. 6A). The detection of  $\beta$ -gal in the cell nucleus was an excellent marker for monitoring fiber cell maturation in the whole lens. We observed that the posterior unstained region was much smaller in the  $\alpha 8^{-/-}$  lenses than in the  $\alpha 8^{+/-}$  lenses at 16.5 dpc (Fig. 7A). Even more surprising, the staining in the cell nuclei was still present in the anterior regions of the interior mature fibers of the  $\alpha 8^{-/-}$  lenses in postnatal day 17 mice, while no staining of the cell nuclei was observed in the same regions of the  $\alpha 8^{+/-}$  sibs (Fig. 7C). The cell nuclei of the  $\alpha 8^{-/-}$  interior fibers were eventually eliminated as the mice reached 3-weeks old (data not shown).

## DISCUSSION

While this work was in progress, White et al. (White et al., 1998) determined the phenotypes of the  $\alpha 8$  knockout mice

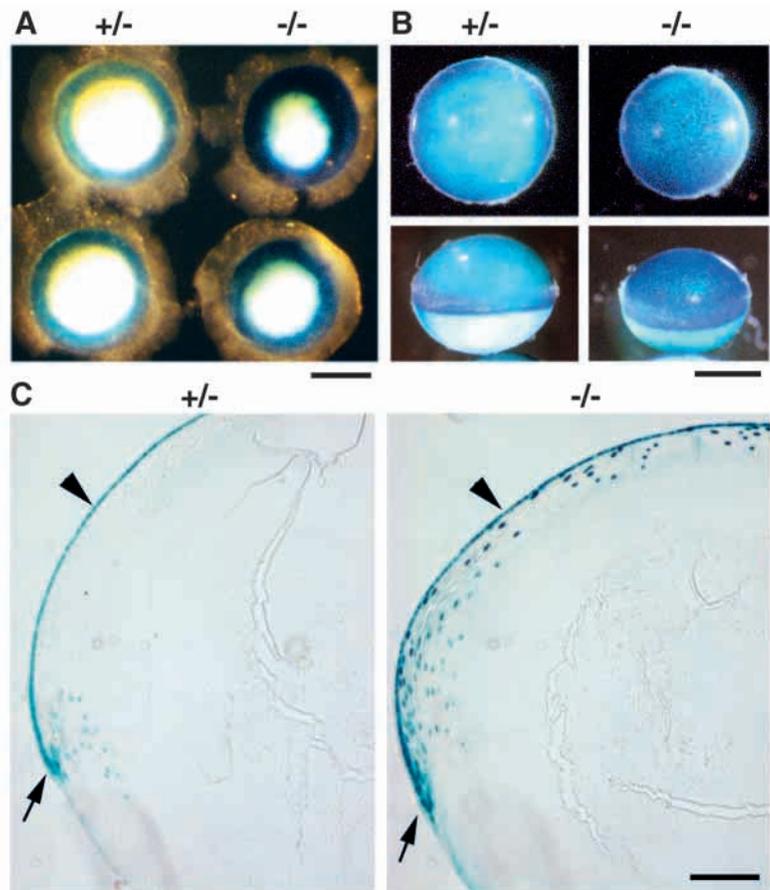


**Fig. 6.** *lacZ* expression patterns in the lenses of  $\alpha 3$  and  $\alpha 8$  knockout mice. (A) A side view of an eye, stained for  $\beta$ -gal, from an  $\alpha 8$  heterozygous ( $+/-$ ) knockout mouse. The lens equator is indicated by an arrow and anterior monolayer epithelium is blue. C, cornea; R, retina; O, optic nerve. B and C show lenses from adult  $\alpha 8^{+/-}$  and  $\alpha 3^{+/-}$  sibs, respectively, stained for  $\beta$ -gal; anterior view. (D) An equatorial view of the  $\alpha 3^{+/-}$  lens in C; an arrow indicates the lens equator. (E-G)  $\alpha 8^{+/-}$ , wild-type ( $+/+$ ) and  $\alpha 3^{+/-}$  embryos at 14 dpc, respectively. (H-J) Magnified views of eyes of the embryos in E, F and G, respectively. Scale bars (A-G) 1 mm; (H-J) 250  $\mu$ m.

using a simple gene targeting strategy without a knock-in *lacZ* reporter gene. These authors also observed microphthalmia with mild nuclear cataracts in their  $\alpha 8^{-/-}$  mice, similar to those in the  $\alpha 8^{-/-}$  mice with a knock-in *lacZ* reporter gene that we generated for this study. Thus, the expression of the knock-in *lacZ* gene had no influence on the phenotypes of  $\alpha 8$  knockout mice, and we were able to carry out a comparative study on both  $\alpha 8$  and  $\alpha 3$  knockouts with a focus on the interactions and coregulations between the two connexin isoforms. Most importantly, by using knock-in *lacZ* as a maturation marker for the denucleation of interior lens fibers, we were able to monitor the transient maintenance of the cell nuclei of interior fibers in the  $\alpha 8^{-/-}$  lenses.

The different phenotypes of the lenses and eyes in  $\alpha 3$  and  $\alpha 8$  knockout mice suggest distinctive functional role(s) for  $\alpha 3$  and  $\alpha 8$  connexins in vivo. Connexin  $\alpha 3$  is essential for

**Fig. 7.** A comparison of the *lacZ* expression patterns in the lenses of  $\alpha 8^{+/-}$  and  $\alpha 8^{-/-}$  sibs.  $\beta$ -gal staining (A) in embryonic lenses from  $\alpha 8^{+/-}$  and  $\alpha 8^{-/-}$  sibs at 16.5 dpc; posterior view; (B) in 4-week old  $\alpha 8^{+/-}$  and  $\alpha 8^{-/-}$  sibs; anterior view (upper panels) and from the equatorial view (lower panels); (C) in sections of lenses from postnatal day 17  $\alpha 8^{+/-}$  (left-hand panel) and  $\alpha 8^{-/-}$  (right-hand panel) sibs. Arrowheads indicate the lens epithelium and arrows point to the bow region of the lenses. Scale bars (A) 0.2 mm; (B) 1 mm; (C) 100  $\mu$ m.



maintaining lens transparency, while  $\alpha 8$  is critical for the growth of the lens. Different expression patterns of  $\alpha 3$  and  $\alpha 8$  connexin genes were also reflected by the expression of the knock-in *lacZ* reporter gene in the lens epithelium of the knockout mice. More importantly, the loss of  $\alpha 8$  connexin was associated with a delayed maturation process of the lens interior fibers, indicated by the retention of the cell nuclei. However, this was not observed in the  $\alpha 3$ -deficient lenses (Gong et al., 1997). The presence of the  $\alpha 8$  connexin in the lens nucleus was dependent on the existence of  $\alpha 3$  connexin. Interestingly, the large size of the gap junction plaques in the cortical fibers was dependent on the presence of  $\alpha 8$  connexin. It is quite clear, then, that this study has demonstrated the importance of both  $\alpha 3$  and  $\alpha 8$  connexin in the lens to ensure its growth and the maintenance of its transparency.

The co-existence of  $\alpha 3$  and  $\alpha 8$  connexins in the lens has been verified in chick (Jiang et al., 1995), sheep (Yang et al., 2000), cattle (Gupta et al., 1994), mouse (Gong et al., 1997), rat (Paul et al., 1991), primate (Lo et al., 1996) and human (Church et al., 1995). Results from both the  $\alpha 3$  and  $\alpha 8$  knockout mice showed that the regulatory machinery of  $\alpha 3$  and  $\alpha 8$  connexin genes operate independently of each other, since there was no compensation in the protein levels of the  $\alpha 8$  connexin when there was a loss of  $\alpha 3$ , and vice versa. Moreover, there was no compensation in the expression of the wild-type allele of  $\alpha 3$  or  $\alpha 8$  connexin in the heterozygous mice, since a 50% reduction in the amount of  $\alpha 8$  or  $\alpha 3$  connexin proteins was detected in the lens homogenates of their heterozygous knockout mice compared with the wild type counterpart. These results suggest that a precise regulatory mechanism exists to control the expression of  $\alpha 3$  and  $\alpha 8$  connexin genes in the lens.

It is very likely that the interactions and regulations between  $\alpha 3$  and  $\alpha 8$  connexins are at the posttranslational level in vivo. Both  $\alpha 3$  and  $\alpha 8$  connexin were detected in the same gap junctional plaques in fiber cells by indirect immunohistochemical staining of frozen sections (Gong et al., 1997) and immuno-gold labeling with an EM replica (Benedetti et al., 2000). The segregation of  $\alpha 3$  connexin from  $\alpha 8$  connexin in lens fibers, which was observed by deconvolution microscopy, supports the notion that lens gap junction plaques consist of a mixture of homomeric  $\alpha 3$  and  $\alpha 8$  channels as well as heteromeric channels. Our recent electrophysiological studies indicated that interior fibers are mainly coupled by homomeric  $\alpha 3$  channels (Baldo et al., 2001). Moreover, the mixing of homomeric gap junction channels, which consist of different connexin isoforms in the

same plaque, has been reported in a cultured cell system (Falk, 2000). According to freeze-fracture graphics and electrophysiological data, both types of knockout mice showed that the  $\alpha 3$  and  $\alpha 8$  connexin were able to form homomeric functional channels in vivo (Gong et al., 1997; Gong et al., 1998; Baldo et al., 2001). Heteromeric connexons have also been demonstrated biochemically in the lens (Konig et al., 1995; Jiang et al., 1996). In addition, homomeric, heteromeric and heterotypic channels formed by  $\alpha 3$  and/or  $\alpha 8$  connexins were characterized in paired *Xenopus* oocytes (Ebihara et al., 1999), as well as in a communication-deficient neuroblastoma (N2A) cell line, using an electrophysiological assay (Hopperstad et al., 2000). These results have indicated that diversified gap junction channels can be formed by the mixing of  $\alpha 3$  and  $\alpha 8$  connexins in vitro.

It is interesting to note that both the  $\alpha 3$  and  $\alpha 8$  connexin demonstrated dependence on one another in a variety of different ways in vivo. For example, the presence of  $\alpha 8$  connexin in the lens nucleus was dependent on the existence of  $\alpha 3$  connexin. This is supported by the fact that no  $\alpha 8$  connexins were detected in the lens nucleus homogenate of the  $\alpha 3^{-/-}$  lenses (Fig. 4), even though the  $\alpha 8$  connexin was able to form functional gap junction channels in the cortical fibers of those lenses (Gong et al., 1998). It is clear that the homomeric  $\alpha 8$  gap junction channel was somehow eliminated in the interior fibers of the  $\alpha 3^{-/-}$  lenses. The mechanism for this is unknown. A simple explanation would be that only the  $\alpha 8$  proteins formed heteromeric channels with the  $\alpha 3$  connexin, or that the homomeric  $\alpha 8$  channels that mixed with

the homomeric  $\alpha 3$  channels in the same junctional plaque (or domain) remained in the normal lens nucleus. It is also possible that the loss of  $\alpha 8$  connexin in the  $\alpha 3$ -/- lens nucleus was due to cataract formation in these lenses. Interestingly, the presence of  $\alpha 3$  connexin in the lens nucleus was independent from  $\alpha 8$  connexin (Fig. 4). Moreover, the  $\alpha 3$  connexin in the  $\alpha 8$ -/- lenses was able to form only the small gap junction plaques, which were much smaller than the gap junction plaques in either the wild-type (Fig. 5) or  $\alpha 3$ -/- lenses (Gong et al., 1997). This suggests that a regulatory mechanism must be present to control the size of gap junction domains (plaques) formed by  $\alpha 3$  and  $\alpha 8$  connexins. As of yet, the mechanism that controls the size of gap junction plaques and the packing of gap junction particles in the plasma membrane has not been elucidated. Studies on either  $\alpha 3$  or  $\alpha 8$  gene knockout mice can verify only the functional role of the remaining homomeric gap junction channels formed by either  $\alpha 3$  or  $\alpha 8$  subunits, and not the heteromeric and heterotypic interactions between the two isoforms. Further studies are, therefore, required to verify the existence of these types of interactions in vivo, as well as to determine the physiological and biological importance of these interactions in the lens.

Surprisingly, we observed a delayed denucleation in the interior fibers of the  $\alpha 8$ -/- lenses. The elimination of the cell nucleus of the interior fibers, as well as the other intracellular organelles, is an essential process for generating lens transparency, and the process is precisely regulated in the lens (Bassnett and Mataic, 1997; Dahm et al., 1998). It has been reported that the denucleation was a part of the apoptotic processes (Wride, 2000). Members of the bcl-2 and caspase families have been reported to be involved in the regulation of nuclear degeneration (Wride et al., 1999), but, the question still remains as to whether this was a simple correlative phenomenon, since the degradation process of the lens nucleus requires days to be completed, while a typical apoptosis only takes minutes to hours. So far, we do not know the molecular interactions linking the loss of  $\alpha 8$  connexin and the denucleation process. It will be crucial to investigate the molecular changes associated with this delayed denucleation in the  $\alpha 8$ -/- lenses. The  $\alpha 8$  knockout mouse will undoubtedly provide a useful system for elucidating the mechanism that controls the maturation of lens fiber cells.

Genetic studies have shown that  $\alpha 3$  and  $\alpha 8$  gene mutations in humans (Shiels et al., 1998; Berry et al., 1999; Mackay et al., 1999; Rees et al., 2000), as well as one point mutant of  $\alpha 8$  in mice (Steele et al., 1998), were linked to semi-dominant cataracts. This is inconsistent with the fact that both  $\alpha 3$  and  $\alpha 8$  knockout mice showed recessive cataractous phenotypes (Gong et al., 1997; White et al., 1998). Therefore, it will be important to generate or identify  $\alpha 3$  and  $\alpha 8$  point mutants with dominant cataracts so that we will be able to try to elucidate the molecular basis for the cataractogenesis linked to connexin mutations in both mice and humans.

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