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Diverse gap junctions modulate distinct mechanisms for fiber cell formation during lens development and cataractogenesis

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Different mutations of α3 connexin (Cx46 or *Gja8*) and α8 connexin (Cx50 or *Gja8*), subunits of lens gap junction channels, cause a variety of cataracts via unknown mechanisms. We identified a dominant cataractous mouse line (*L1*), caused by a missense α8 connexin mutation that resulted in the expression of α8-S50P mutant proteins. Histology studies showed that primary lens fiber cells failed to fully elongate in heterozygous $\alpha8^{S50P/+}$ embryonic lenses, but not in homozygous $\alpha8^{S50P/S50P}$, $\alpha8^{-/-}$ and $\alpha3^{-/-}$ $\alpha8^{-/-}$ mutant embryonic lenses. We hypothesized that α8-S50P mutant subunits interacted with wild-type α3 or α8, or with both subunits to affect fiber cell formation. We found that the combination of mutant α8-S50P and wild-type α8 subunits specifically inhibited the elongation of primary fiber cells, while the combination of α8-S50P and wild-type α3 subunits disrupted the formation of secondary fiber cells. Thus, this work provides the first in vivo evidence that distinct mechanisms, modulated by diverse gap junctions, control the formation of primary and secondary fiber cells during lens development. This explains why and how different connexin mutations lead to a variety of cataracts. The principle of this explanation can also be applied to mutations of other connexin isoforms that cause different diseases in other organs.

KEY WORDS: Cataract, Connexin, Gap junction, Lens fiber cell

INTRODUCTION

Cataracts, defined as lens opacities, are the leading cause of blindness in the world. To date, cataract surgery remains the only way to cure them and there is no non-surgical method to delay or prevent cataractogenesis. Although decades of studies have significantly improved our understanding of risk factors for developing cataracts, as well as biochemical and morphological changes that occur during cataract formation, the mechanisms for how the lens establishes and maintains its transparency remain largely unknown. The lens is formed through sequential events including the differentiation and elongation of posterior lens vesicle cells to form primary fibers that fill the lumen of lens vesicle, followed by the differentiation and elongation of anterior lens epithelial cells to form secondary fiber cells that lie on top of primary fibers at the lens periphery. Cell elongation is a hallmark of lens fiber differentiation during lens development (Bassnett, 2004; Piatigorsky, 1981). A mechanistic understanding of fiber cell elongation in vivo has relied on speculations based on in vitro studies of cultured lens epithelial cells. Moreover, mechanisms that differentially regulate lens primary and secondary fiber cell formation are unknown.

Lens fiber cells are coupled by intercellular gap junction channels formed by α3 (connexin 46) and α8 (connexin 50) connexin subunits to maintain the homeostasis required for lens transparency (Goodenough, 1992; Mathias et al., 1997). Mutations in the α3 (*Gja3*) and α8 (*Gja8*) connexin genes are one of the common causes for inherited cataracts in humans and mice. Connexin proteins have four transmembrane domains with three intracellular regions (the N terminus, a cytoplasmic loop and the C terminus) and two extracellular loops (E1 and E2) (Yeager and Nicholson, 2000). Six connexin subunits oligomerize to form one connexon (hemichannel). A gap junction channel is formed by the docking of extracellular loops of two opposing connexons in the plasma membrane. Hundreds of gap junction channels come together to form gap junctions that are morphologically defined as specialized punctate 'plaques' of cell-to-cell contacts. These channels with small pores provide pathways for the direct exchange of small molecules between adjacent cells (Fleishman et al., 2004; Unger et al., 1999). Co-expression of two types of connexin subunits in cells will allow the formation of homomeric connexons (consisting of one type of subunit), heteromeric connexons (consisting of two types of subunits), homotypic channels (the docking of two identical connexons) and heterotypic channels (the docking of two different types of connexons) (Kumar and Gilula, 1996).

Studies of $\alpha3^{-/-}$ knockout mice have suggested that α3 connexin is essential for maintaining lens transparency (Gong et al., 1997), while the analyses of $\alpha8^{-/-}$ knockout mice and knock-in α3(50KI46/50KI46) mice have revealed that α8 connexin is important for lens growth (Martinez-Wittinghan et al., 2003; Martinez-Wittinghan et al., 2004; Rong et al., 2002; White et al., 1998). Although $\alpha8^{-/-}$ lenses show reduced epithelial proliferation and delayed fiber cell maturation, the mechanism for how the loss of α8 connexin leads to smaller lenses is unknown (Rong et al., 2002; Sellitto et al., 2004). The interaction between α3 and α8 subunits has been suggested by their colocalization in the fiber cells of different vertebrate lenses and the biochemical isolation of heteromeric connexons from the lens (Gong et al., 1997; Jiang and Goodenough, 1996; Konig and Zampighi, 1995; Lo et al., 1996). The α3 and α8 connexin subunits are able to form heteromeric and

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heterotypic channels in paired *Xenopus* oocytes and cultured cells in vitro (Hopperstad et al., 2000; White et al., 1994). Thus, diverse gap junctions formed by α3 and α8 subunits need to be further investigated in vivo.

The roles of diverse gap junctions have never been elucidated during lens development due to a lack of an appropriate experimental model in vivo. We hypothesize that dominant cataracts are caused by altered intercellular communication mediated by diverse gap junction channels consisting of mutant and wild-type connexin subunits in the lens. In this work, we have found that the combination of mutant α8-S50P subunits (a mutation in the extracellular loop 1) and wild-type α8 subunits specifically inhibits the elongation of embryonic lens fiber cells, while the combination of mutant α8-S50P and wild-type α3 subunits disrupts the differentiation and elongation of postnatal lens fibers. This work reveals that diverse gap junctions mediate distinct mechanisms to control the formation of lens primary and secondary fiber cells, and this also explains why and how a variety of cataracts can result from perturbations of different types of gap junctions during lens development.

MATERIALS AND METHODS

Mouse ENU mutation, genomic linkage analysis and causative gene identification

Generation of ENU-mutagenized mice, mouse breeding and genome-wide linkage analysis were described previously (Du et al., 2004). The dominant *L1* mutation, in the C57BL/6J (B6) strain background, was outcrossed with wild-type C3H/Hej mice to produce affected G1 hybrid mice that were further crossed with wild-type C3H/Hej mice to produce second generation (G2) mice. The G2 mice were phenotyped with a slit lamp and genotyped with genomic DNA for a genome-wide linkage analysis by using a total of 59 microsatellite markers (Du et al., 2004). Based on the chromosomal location, causative gene candidates were identified from the Mouse Genome Database at the National Center for Biotechnology Information (NCBI) website. PCR fragments of the α8 connexin gene were amplified from mutant genomic DNA isolated from homozygous mutant mice, as previously described (Chang et al., 2002), and followed by DNA sequencing analysis.

Examination of lens phenotypes

Mouse pupils were dilated by using an eye drop containing 1% phenylephrine and 1% atropine before lens clarity was examined using a slit lamp. The cataract was directly imaged in living animals by a slit lamp (Nikon-FS3) using a camera and Kodak elite2 200ASA color slide films. Fresh lenses, dissected from enucleated eyeballs of wild-type and mutant mice, were imaged under a Leica MZ16 dissecting scope using a digital camera.

Generation of compound mutant mice

L1 heterozygous $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ mice were bred with $\alpha8^{-/-}$ $\alpha3^{-/-}$ double knockout mice to produce the $\alpha8^{S50P/-}$ $\alpha3^{+/-}$ and $\alpha8^{+/-}$ $\alpha3^{+/-}$ mutant mice. Both *L1* homozygous $\alpha8^{S50P/S50P}$ $\alpha3^{+/+}$ and double mutant $\alpha8^{S50P/S50P}$ $\alpha3^{-/-}$ mice were generated from the intercross of $\alpha8^{S50P/-}$ $\alpha3^{+/-}$ mice. Double mutant $\alpha8^{S50P/S50P}$ $\alpha3^{-/-}$ mice were bred with $\alpha3^{-/-}$ knockout mice to produce $\alpha8^{S50P/+}$ $\alpha3^{-/-}$ mice. Previously described PCR methods were used to distinguish α3 or α8 knockout alleles from wild-type or mutant α8 alleles (Chang et al., 2002).

Histology, thin-section TEM and immunohistochemistry

Enucleated eyeballs were fixed in a solution containing 2% glutaraldehyde and 2.5% formaldehyde in 0.1 M cacodylate buffer (pH 7.2) at room temperature for at least 5 days and were postfixed in 1% aqueous OsO_4, stained en bloc with 2% aqueous uranyl acetate and then dehydrated through graded acetone. Samples were embedded in Epon resin (Ted Pella, Redding, CA). Sections (1 μm) were collected on glass slides and stained with Toluidine Blue. Bright-field images were acquired via a Zeiss Axiovert 200 light microscope with a digital camera. Thin sections (80 nm) were cut with a diamond knife, stained with 5% uranyl acetate followed by Reynold's lead citrate before examination under a JEOL JEM-1200EX electron microscope (JEOL, Tokyo, Japan).

A previously described method was used to prepare lens frozen sections for immunohistochemical analysis with different antibodies (Gong et al., 1997). The following reagents were used for immunostaining embryonic lens frozen sections: a rabbit polyclonal antibody against the C-terminal region of α8 connexin (generously provided by Dr M. J. Wolosin at Mount Sinai School of Medicine, New York), rhodamine phalloidin (Molecular Probe) for detecting F-actin and DAPI (Vector Laboratories) for labeling cell nuclei. Fluorescent images were collected under a Zeiss Axiovert 200 fluorescent microscope with an Axiocam camera or a Leica laser confocal microscope with a high resolution digital camera.

RESULTS

The α8-S50P point mutation causes dominant cataracts in *L1* mutant mice

A male cataractous mouse was identified from ENU-mutagenized mice by slit-lamp examination. This male founder was bred with wild-type C57BL/6J (B6) female mice, and half of the offspring developed a phenotype identical to the male founder. This mutant line was named lens mutation 1 (*L1*) and was maintained in the B6 background (Fig. 1A). The *L1* heterozygous mice developed whole cataracts with small eyes (about 30% of the size of wild-type eyes) at weaning age (Fig. 1A,B). Genomic DNA samples of 30 normal and 36 affected G2 mice were used for a genome-wide linkage analysis. The *L1* mutation was mapped to mouse chromosome 3 with a Lod score of 6.8 at the linkage marker D3Mit98, which is in the vicinity of the *Gja8* (α8 connexin) gene (Fig. 1C).

It is known that α8 connexin mutations cause cataracts in both humans and mice. Therefore, we performed DNA sequencing analysis using PCR fragments amplified from the genomic DNA of *L1* homozygous mice. We found a missense mutation (T→C) of the *Gja8* gene, resulting in the replacement of the serine residue at codon 50 by a proline residue (S50P) in the extracellular loop 1 (E1-loop) of the α8 connexin protein (Fig. 1D). Thus, the dominant cataracts in the *L1* mouse line are caused by the α8-S50P mutation. The genotypes for *L1* heterozygous and homozygous mice are labeled as $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ and $\alpha8^{S50P/S50P}$ $\alpha3^{+/+}$, respectively. The wild-type, homozygous α8 knockout and α8/α3 double knockout mice are labeled as $\alpha8^{+/+}$ $\alpha3^{+/+}$, $\alpha8^{-/-}$ $\alpha3^{+/+}$ and $\alpha8^{-/-}$ $\alpha3^{-/-}$.

We further confirmed that the $\alpha8^{S50P/S50P}$ $\alpha3^{+/+}$ mice developed small and ruptured cataractous lenses similar to the $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ mice at weaning age (Fig. 2A,B). Histological data showed severely disorganized fiber cells, vacuole formation and posterior capsule rupture in both $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ and $\alpha8^{S50P/S50P}$ $\alpha3^{+/+}$ lenses (Fig. 2C,D). Thus, secondary fiber cell formation was severely disrupted in both $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ and $\alpha8^{S50P/S50P}$ $\alpha3^{+/+}$ postnatal lenses.

Primary fiber cell elongation is inhibited only in the $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ lenses

As altered fiber cells were obviously observed in the neonatal lenses of both $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ and $\alpha8^{S50P/S50P}$ $\alpha3^{+/+}$ mice (data not shown), we carried out histological studies of their embryonic lenses. At 15.5 days post-conception (E15.5), both $\alpha8^{+/+}$ $\alpha3^{+/+}$ and $\alpha8^{-/-}$ $\alpha3^{+/+}$ embryonic lenses showed no space between the primary fibers and overlying anterior epithelium (Fig. 3A,B). Unexpectedly, a large cystic lumen between posterior primary fiber cells and anterior epithelium was observed in the E15.5 $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ embryonic lenses with 100% penetrance (Fig. 3C), but not in the E15.5 $\alpha8^{S50P/S50P}$ $\alpha3^{+/+}$ embryonic lenses (Fig. 3D). Histology data further

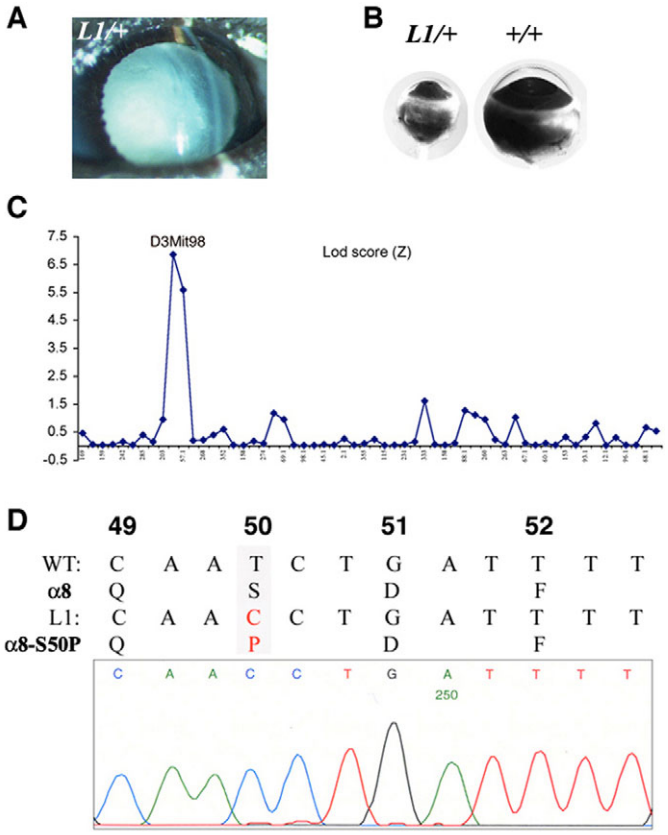

Fig. 1. A missense mutation of *Gja8* (α8-connexin or Cx50) leads to whole cataracts and microphthalmia in the *L1* mutant mice. (**A**) A slit-lamp photo shows the severe cataract of a heterozygous mutant (*L1*/+) mouse at the age of 1 month. (**B**) The eyeballs of heterozygous mutant (*L1*/+) mice are about 30% of the size of wild-type (+/+) controls at the age of 3 weeks. (**C**) Genome-wide linkage analysis shows that the *L1* mutation is mapped to chromosome 3 near marker D3Mit98 with a Lod score of 6.8, which is in the vicinity of *Gja8* gene. (**D**) DNA sequencing of homozygous *L1* mouse genomic DNA shows a missense mutation in the α8 connexin gene. A change of T to C at position 148 causes a substitution of the serine residue at codon 50 by a proline residue (S50P).

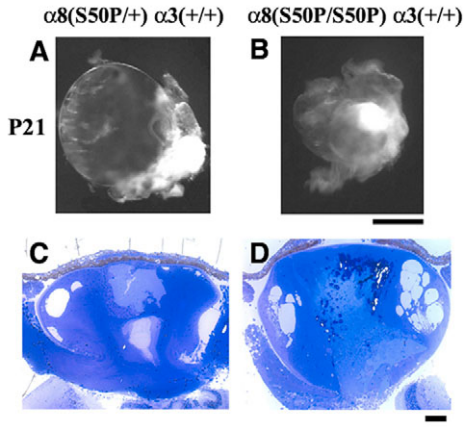

Fig. 2. Both heterozygous $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ and homozygous $\alpha8^{S50P/S50P}$ $\alpha3^{+/+}$ lenses display lens rupture and severely altered fiber cells at weaning age. (**A**) Lens from an $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ mouse at P21. (**B**) Lens from a P21 $\alpha8^{S50P/S50P}$ $\alpha3^{+/+}$ mouse. (**C**,**D**) Toluidine Blue-stained lens sections show posterior rupture and severely altered fiber cells in the $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ (C) and $\alpha8^{S50P/S50P}$ $\alpha3^{+/+}$ (D) lenses of P21 mice. Scale bars: 0.5 mm in the upper panels; 100 μm in the lower panels.

verified that at earlier stages (E13.5), posterior primary fiber cells did not reach the anterior epithelium in the $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ lenses, while primary fiber cells in wild-type lenses elongated and obliterated the lumen of the lens vesicle at the same embryonic stage (data not shown).

Thus, although mature fiber cells are severely altered in both postnatal day 21 (P21) $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ and $\alpha8^{S50P/S50P}$ $\alpha3^{+/+}$ lenses (Fig. 2), the elongation of primary fiber cells is significantly perturbed only in $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ embryonic lenses, not in $\alpha8^{S50P/S50P}$ $\alpha3^{+/+}$ and $\alpha8^{-/-}$ $\alpha3^{+/+}$ lenses during embryonic development. We hypothesize that α8-S50P is a gain-of-function mutant subunit, as neither a loss-of-function of α8 connexin in $\alpha8^{-/-}$ $\alpha3^{+/+}$ lenses nor a normal function of α8 connexin in wild-type $\alpha8^{+/+}$ $\alpha3^{+/+}$ lenses inhibits primary fiber cell elongation. Moreover, normal elongation of primary fiber cells in $\alpha8^{S50P/S50P}$ $\alpha3^{+/+}$ lenses suggests that an interaction between mutant α8-S50P subunits and endogenous wild-type α8 subunits is probably required for the suppression of primary fiber cell elongation in $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ embryonic lenses.

We hypothesize that the gain-of-function α8-S50P mutation alters the intercellular communication in lens fiber cells by interacting with endogenous wild-type α8 and/or α3 subunits to form mutant gap junctions. To test this hypothesis, we examined the presence of gap junctions in $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ embryonic lenses. A representative immunostaining image showed typical punctate fluorescent spots of α8 connexin in an E15.5 wild-type lens frozen section detected by an anti-α8 antibody (Fig. 3E). Similar fluorescent signals were also detected in posterior fiber cells of E15.5 $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ lens sections (Fig. 3F,G). Transmission electron microscope (TEM) analysis further confirmed the presence of bona fide gap junctions in $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ lenses (Fig. 3H). Therefore, we investigated the subunit composition of mutant gap junctions that perturb the formation of primary and secondary fiber cells in α8-S50P mutant lenses by a genetic approach.

The combination of wild-type α8 and mutant α8-S50P subunits inhibits the elongation of lens primary fibers

In order to determine the subunit composition of mutant gap junction channels that mediate the inhibition of primary fiber cell elongation in $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ embryonic lenses, we replaced wild-type α3 and α8 alleles with the null alleles. By breeding the $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ mutant mice with the $\alpha3^{-/-}$ knockout and $\alpha8^{-/-}$ knockout mice, we have generated two different compound mutant mice: $\alpha8^{S50P/+}$ $\alpha3^{-/-}$ mice that lack wild-type α3 connexin and $\alpha8^{S50P/-}$ $\alpha3^{+/+}$ mice that lack wild-type α8 connexin. Histological data showed a cystic lumen only in $\alpha8^{S50P/+}$ $\alpha3^{-/-}$ embryonic lenses (Fig. 4A), but not in $\alpha8^{S50P/-}$ $\alpha3^{+/+}$ lenses (Fig. 4B). Gap junctions were also detected in the fiber cells of these compound mutant embryonic lenses by immunohistochemistry and TEM (data not shown). These results suggest that α8-S50P subunits interact with endogenous wild-type α8 subunits to inhibit primary fiber cell elongation, while the presence of endogenous wild-type α3 subunits does not affect primary fiber formation. Thus, it is possible that α8-S50P and wild-type α8 subunits form mutant gap junction channels that modulate a unique mechanism essential for the elongation of lens primary fiber cells.

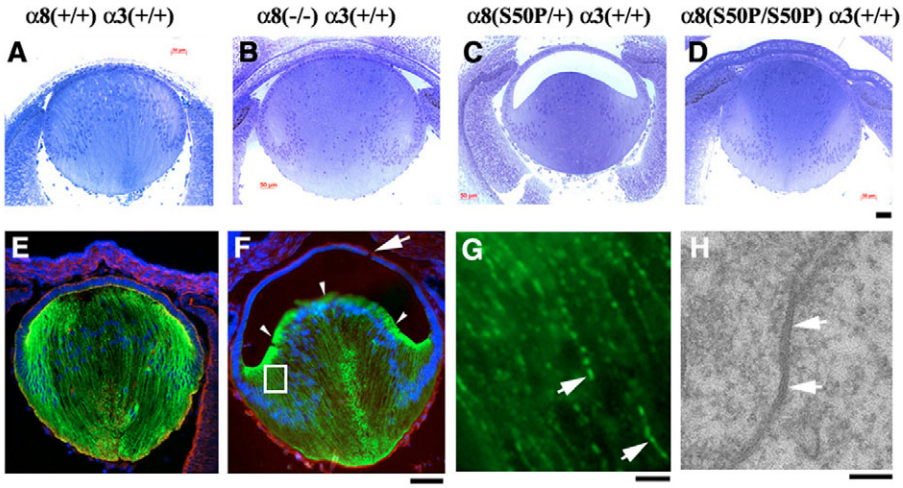

Fig. 3. Primary fiber cells fail to fully elongate to contact the anterior epithelium only in $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ embryonic lenses even though typical gap junctions are still present. (**A**,**B**,**D**) Wild-type, $\alpha8^{-/-}$ knockout and $\alpha8^{S50P/S50P}$ $\alpha3^{+/+}$ embryonic lenses do not have any space between the epithelium and the underlying fiber cells at E15.5. (**C**) An E15.5 $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ embryonic lens shows a large cystic lumen between posterior primary fiber cells and the anterior epithelium. All images were taken from Toluidine Blue-stained embryonic sections. (**E**,**F**) Triple-labeled images of α8 connexin (green), F-actin (red) and nuclei (blue) of frozen sections from wild-type (E) and $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ (F) embryonic lenses, respectively. The punctate green fluorescent spots represent typical staining of α8 connexins in lens fiber cells. A large cystic lumen is present only in the $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ embryonic lens in F. White arrowheads indicate the anterior ends of posterior fiber cells and a large white arrow indicates the anterior epithelium. (**G**) A high magnification image of the selected area in F shows punctate fluorescent spots (indicated by white arrows). (**H**) TEM shows bona fide gap junctions (indicated by white arrows) between posterior fiber cells of the E15.5 $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ lens. Scale bars: 50 μm for A-D; 100 μm for E,F; 5 μm for G; 100 nm for H.

The combination of wild-type α3 and mutant α8-S50P subunits disrupts the formation of postnatal secondary fibers

In order to understand why both $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ and $\alpha8^{S50P/S50P}$ $\alpha3^{+/+}$ mice developed whole cataracts at weaning age (Fig. 2), we further examined the postnatal lens phenotypes of $\alpha8^{S50P/+}$ $\alpha3^{-/-}$ and $\alpha8^{S50P/-}$ $\alpha3^{+/+}$ mice. Surprisingly, the $\alpha8^{S50P/+}$ $\alpha3^{-/-}$ mice developed a nuclear cataract rather than a whole cataract. A lens from a P21 $\alpha8^{S50P/+}$ $\alpha3^{-/-}$ mouse revealed a nuclear cataract with a transparent cortex (Fig. 4E). Histological data showed degenerated nuclear fibers but normal peripheral cortical fibers in the P14 $\alpha8^{S50P/+}$ $\alpha3^{-/-}$ lens (Fig. 4C). By contrast, $\alpha8^{S50P/-}$ $\alpha3^{+/+}$ mice developed microphthalmia with whole cataracts similar to the $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ and $\alpha8^{S50P/S50P}$ $\alpha3^{+/+}$ mice. A lens from a P21 $\alpha8^{S50P/-}$ $\alpha3^{+/+}$ mouse was small and ruptured with a cataract (Fig. 4F). Disrupted secondary fiber cells, enlarged vacuole-like extracellular spaces and posterior capsule rupture were observed in the P14 $\alpha8^{S50P/-}$ $\alpha3^{+/+}$ lens (Fig. 4D).

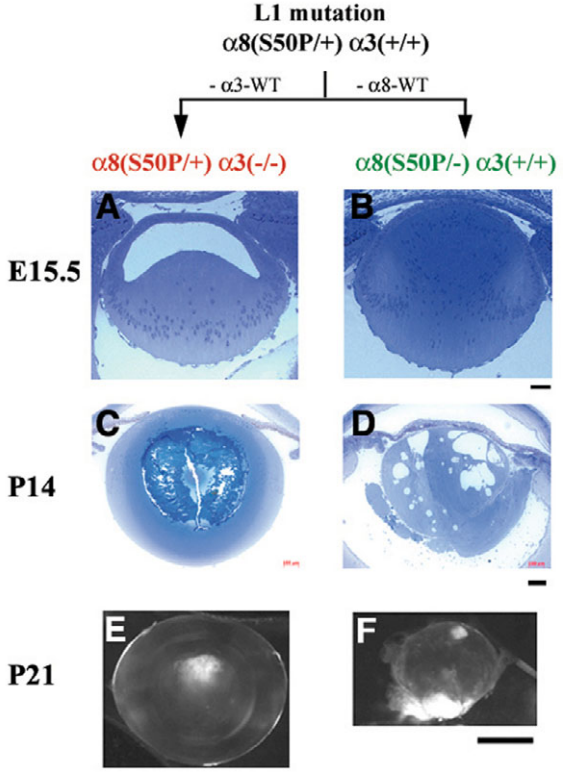

Fig. 4. The generation and characterization of $\alpha8^{S50P/+}$ $\alpha3^{-/-}$ and $\alpha8^{S50P/-}$ $\alpha3^{+/+}$ compound mutant mice. (**A**) Wild-type α3 connexins were eliminated by breeding the $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ mice with the $\alpha3^{-/-}$ knockout mice. Toluidine Blue-stained section of an E15.5 $\alpha8^{S50P/+}$ $\alpha3^{-/-}$ embryonic lens shows a large lumen between posterior primary fiber cells and the anterior epithelium, similar to the $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ embryonic lens section in Fig. 3C. (**B**) Wild-type α8 connexins were eliminated by breeding the $\alpha8^{S50P/+}$ $\alpha3^{+/+}$ mice with the $\alpha8^{-/-}$ knockout mice. An E15.5 $\alpha8^{S50P/-}$ $\alpha3^{+/+}$ lens section shows no space between the epithelium and posterior primary fiber cells. (**C**) A P14 $\alpha8^{S50P/+}$ $\alpha3^{-/-}$ lens section shows degenerated primary fibers in the lens nucleus with relatively normal peripheral secondary fibers. (**D**) A Toluidine Blue-stained P14 $\alpha8^{S50P/-}$ $\alpha3^{+/+}$ lens section shows severe disruption of fiber cells and ruptured posterior capsule. (**E**) Lens from an $\alpha8^{S50P/+}$ $\alpha3^{-/-}$ mouse at P21 shows a nuclear cataract but a relatively normal cortex. (**F**) A lens from a P21 $\alpha8^{S50P/-}$ $\alpha3^{+/+}$ mouse shows a small and ruptured lens with cataract. Scale bars: 50 μm for E15.5 sections; 100 μm for P14 sections; 0.5 mm for P21 lenses.

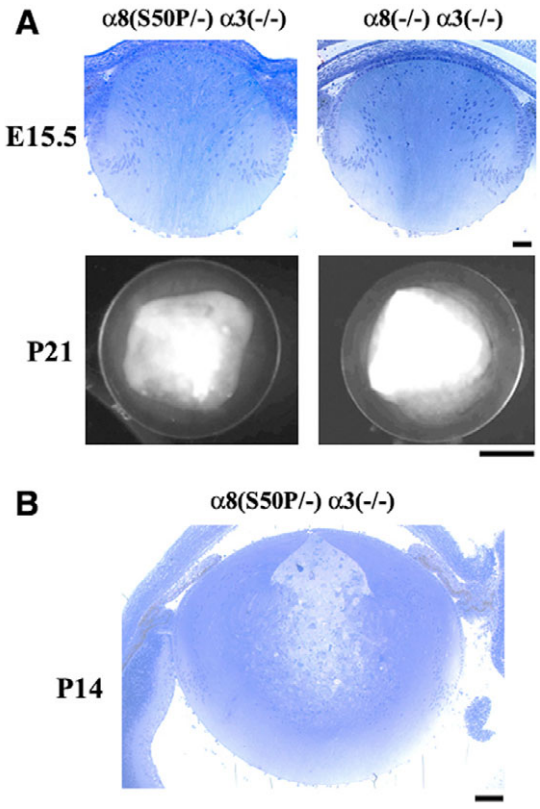

Fig. 5. A comparison of $\alpha8^{S50P/-}$ $\alpha3^{-/-}$ and $\alpha8^{-/-}$ $\alpha3^{-/-}$ lenses. (**A**) Toluidine Blue-stained E15.5 $\alpha8^{S50P/-}$ $\alpha3^{-/-}$ and $\alpha8^{-/-}$ $\alpha3^{-/-}$ embryonic lens sections show no space between the epithelium and posterior primary fiber cells (upper panels). Photos of P21 $\alpha8^{S50P/-}$ $\alpha3^{-/-}$ and $\alpha8^{-/-}$ $\alpha3^{-/-}$ lenses display large nuclear cataracts with transparent cortex (lower panels). (**B**) A Toluidine Blue-stained P14 $\alpha8^{S50P/-}$ $\alpha3^{-/-}$ lens section shows degenerating inner fiber cells and normal peripheral secondary fibers. Scale bars: 50 μm for E15.5 sections; 0.5 mm for P21 lenses; 100 μm for P14 sections.

These data confirm that the combination of endogenous wild-type α3 and mutant α8-S50P subunits disrupts the formation of postnatal lens secondary fibers, while the presence of endogenous wild-type α8 connexin does not affect secondary fiber formation. These results also provide a molecular explanation for why the *L1* heterozygous ($\alpha8^{S50P/+}$ $\alpha3^{+/+}$) and *L1* homozygous ($\alpha8^{S50P/S50P}$ $\alpha3^{+/+}$) mice developed similar phenotypes, such as ruptured lenses and microphthalmia, at the weaning age. Thus, it is possible that α8-S50P and wild-type α3 subunits form mutant gap junction channels that modulate a different mechanism for regulating proper formation of secondary fibers in postnatal lenses.

Mutant α8-S50P subunits alone have no effect on the formation of lens primary or secondary fiber cells

We have also generated $\alpha8^{S50P/-}$ $\alpha3^{-/-}$ mutant mice that lack both endogenous wild-type α8 and α3 connexins and compared their lens phenotypes with those of double knockout $\alpha8^{-/-}$ $\alpha3^{-/-}$ mice. Histological data revealed that both $\alpha8^{S50P/-}$ $\alpha3^{-/-}$ and $\alpha8^{-/-}$ $\alpha3^{-/-}$ embryonic lenses had normal elongation of primary fiber cells, and both mutant mice developed large nuclear cataracts at the age of three weeks (Fig. 5A). A histological section of a P14 $\alpha8^{S50P/-}$ $\alpha3^{-/-}$ lens displayed normal secondary fiber cells in lens periphery but degenerated inner mature fiber cells (Fig. 5B). Lens phenotypes of different types of α8-S50P mutant mice are summarized in Table 1. In summary, these data suggest that mutant α8-S50P subunits probably have no function in vivo and that the interactions between these mutant subunits and endogenous wild-type α8 or α3 connexins perturb primary fiber cell elongation or secondary fiber cell formation, respectively.

DISCUSSION

Intercellular gap junction channels formed by α1 (Cx43), α3 (Cx46) and α8 (Cx50) subunits have been suggested to provide a sophisticated regulatory network to coordinate lens growth and to maintain lens transparency throughout life (Goodenough, 1992). Lens fiber cells are coupled by gap junction channels consisting of α3 and α8 connexin subunits. This work demonstrates that the combination of α8-S50P and endogenous wild-type α8 subunits specifically inhibits the elongation of primary fiber cells in embryonic lenses, while the combination of α8-S50P and endogenous wild-type α3 subunits disrupts the proper formation of secondary fiber cells in postnatal lenses. These results suggest that gap junctions formed by α8-S50P and wild-type α8 subunits alter a unique mechanism required for the elongation of lens primary fiber cells, while gap junctions formed by α8-S50P and wild-type α3 subunits perturb a separate and distinct mechanism needed for proper formation of secondary fiber cells in postnatal lenses. Thus, this work provides the first in vivo evidence for a working model that diverse gap junction communications modulate different signaling mechanisms for primary fiber cell elongation or secondary fiber cell formation during lens development (Fig. 6).

This work provides an in vivo model to understand a fundamental mechanism for why and how diverse gap junction channels are used in almost all organs supported by the fact that two or more types of connexin subunits are commonly co-expressed in cells (Goodenough et al., 1996; Kumar and Gilula, 1996). It is generally accepted that diverse gap junctions provide a broad spectrum of pathways to ensure the homeostasis needed for various cellular functions in vivo. However, studies to define the roles of these diverse gap junctions have previously been hindered by an inability to distinguish gap junction channels formed by different types of subunits from the channels formed by one type of subunits in vivo.

Table 1. Summary of lens phenotypes of different α8-S50P mutant mice at 3 weeks of age

Mouse line/genotype	Pimary fiber/secondary fiber	Lens clarity/morphology/size
L1 heterozygous/$\alpha8^{S50P/+}$ $\alpha3^{+/+}$	Elongation inhibition/disruption	Whole cataract/posterior rupture/very small
L1 homozygous/$\alpha8^{S50P/S50P}$ $\alpha3^{+/+}$	Normal elongation/disruption	Whole cataract/posterior rupture/very small
L1 heter w/o α3/$\alpha8^{S50P/+}$ $\alpha3^{-/-}$	Elongation inhibition/normal	Nuclear cataract/no rupture/small
L1 heter w/o α8/$\alpha8^{S50P/-}$ $\alpha3^{+/+}$	Normal elongation/disruption	Whole cataract/posterior rupture/very small
L1 heter w/o α3 & α8/$\alpha8^{S50P/-}$ $\alpha3^{-/-}$	Normal elongation/normal*	Large nuclear cataract/no rupture/small

*Like double homozygous knockout $\alpha8^{-/-}$ $\alpha3^{-/-}$ lenses, $\alpha8^{S50P/-}$ $\alpha3^{-/-}$ lenses display normal peripheral secondary fiber cells, but inner mature fiber cells undergo degeneration to form a large nuclear cataract.

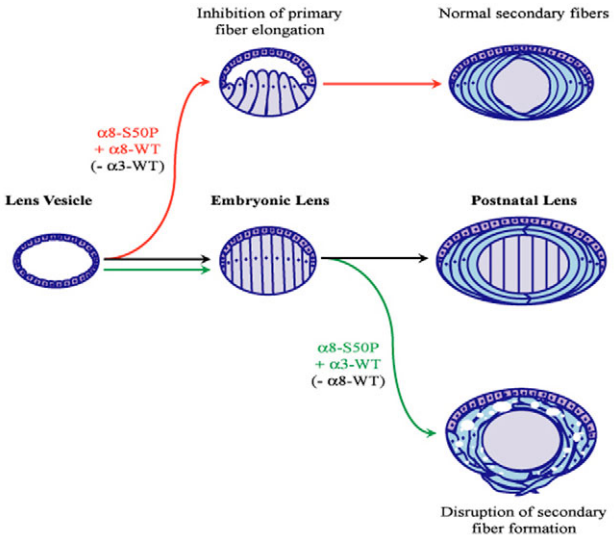

Fig. 6. A model for the cataract formation produced by α8-S50P mutation. Normal embryonic and postnatal lenses are formed in the presence of wild-type α8 (α8-WT) and α3 (α3-WT) connexins (following the black arrows). In the absence of α3-WT, the interaction between α8-S50P mutant connexin (α8-S50P) and α8-WT suppresses the elongation of primary fiber cells in embryonic lenses but does not affect the formation of secondary fiber cells in postnatal lenses (following the red arrows). In the absence of α8-WT, the α8-S50P mutant connexin interacts with endogenous α3-WT to disrupt the formation of secondary fiber cells in postnatal lenses but does not affect the elongation of primary fiber cells (following the green arrows).

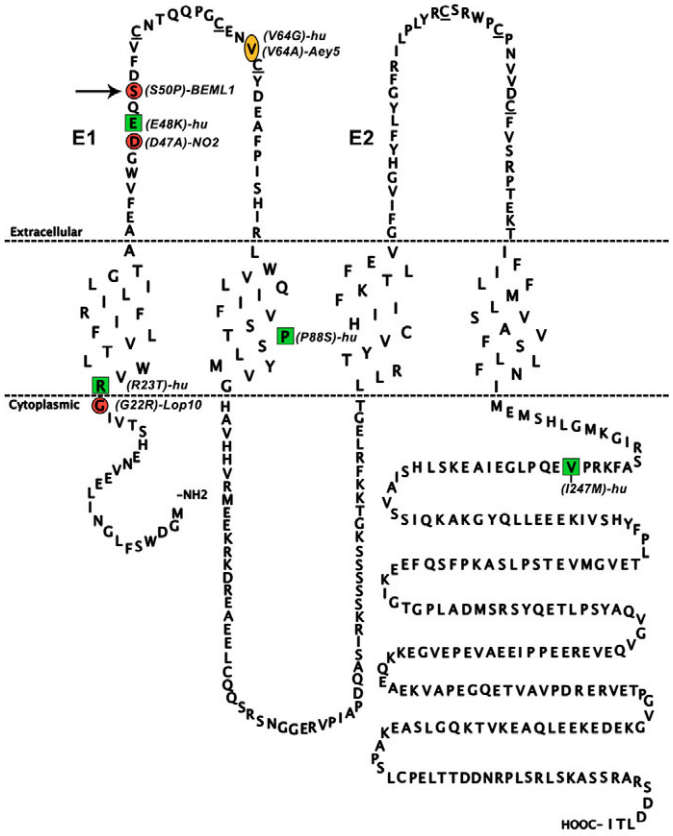

Fig. 7. An illustration of α8 connexin (Cx50) protein topology. Known human mutations are indicated by green squares (R23T, E48K, P88S, I247M) and an orange oval (V64G) (Shiels et al., 1998; Berry et al., 1999; Polyakov et al., 2001; Willoughby et al., 2003; Zheng et al., 2005), and known mouse mutations are indicated by red circles (G22R, D47A, and S50P) and an orange oval (V64A) (Chang et al., 2002; Graw et al., 2001; Steele et al., 1998). An arrow indicates the S50P mutation.

Previous results from studies of loss-of-function knockout mice suggest that α3 connexin is essential for maintaining lens transparency, while α8 connexin is required for lens growth. Functional differences between channels consisting of mixed α8 and α3 subunits and channels consisting of either α8 or α3 subunits in vivo are unclear (Martinez-Wittinghan et al., 2003). Studies of the α8-G22R mutation (a mutation in the N-terminal domain) have demonstrated that severe lens phenotypes are partly caused by the mutant gap junction channels consisting of both mutant α8-G22R and endogenous wild-type α3 subunits (Chang et al., 2002). The α8-G22R mutant probably acts as a dominant-negative inhibitor to perturb gap junction communication by oligomerizing with wild-type α8 and/or α3 subunits. This predication is also supported by the fact that the elongation of primary fiber cells is normal in both α8-G22R heterozygous and homozygous embryonic lenses (C.-h.X, D.C. and X.G., unpublished), and in $\alpha8^{-/-}$ $\alpha3^{-/-}$ lenses without gap junction channels. Therefore, based on the result that a combination of α8-S50P and wild-type α8 subunits inhibits the elongation of primary fiber cells, we propose that α8-S50P mutant proteins are gain-of-function subunits that perturb the intercellular gap junction communication or possibly hemichannels by interacting with wild-type α8 subunits. A gain-of-function approach has never been used to investigate the roles of intercellular gap junction communications in vivo. The downstream signaling mechanisms modulated by diverse intercellular gap junctions formed by α8-S50P and α8 or α3 connexins in the lens are unknown. Thus, the α8-S50P mutation provides a useful experimental model with which to further investigate the mechanisms that uniquely regulate the elongation of primary fibers and the formation of secondary fiber cells during lens development.

To date, six α3 mutants (F32L, P59L, N63S, P187L, S380fs and N188T) and five α8 mutants (R23T, E48K, P88S, I247M and V64G) have been linked to dominant cataracts in humans (Bennett et al., 2004; Berry et al., 1999; Jiang et al., 2003; Li et al., 2004; Mackay et al., 1999; Polyakov et al., 2001; Rees et al., 2000; Shiels et al., 1998; Willoughby et al., 2003; Zheng et al., 2005). In addition, three α8 mutants (G22R, D47A and V64A) cause different dominant cataracts in mice (Chang et al., 2002; Graw et al., 2001; Steele et al., 1998). Previously reported mutations of α8 connexin are illustrated in a topological model of α8 protein (Fig. 7). These mutations cause a variety of dominant cataracts in humans and mice. A previous study of human α8-P88S mutant (located in the second transmembrane domain) suggests a hypothesis that the dominant cataract is caused by cellular defects triggered by mistrafficking and intracellular accumulation of toxic aggregates composed of mutant connexin proteins (Berthoud et al., 2003). However, this hypothesis cannot explain the cataracts caused by the α8-S50P mutation and the α8-G22R mutation. Data from our current study of the α8-S50P mutation and our previous study of the α8-G22R mutation reveal that different types of cataracts result from a perturbation of intercellular gap junction communication by the specific interactions between mutant subunits and endogenous wild-type subunits during lens

development. Therefore, our current work demonstrates a new mechanistic explanation for why and how different connexin mutations lead to a variety of cataracts. Moreover, this new mechanism explains how mutations of other connexin isoforms cause different diseases in other organs, such as a recent finding that the Cx43-G60S mutation in extracellular loop 1 causes oculodentodigital dysplasia (Flenniken et al., 2005). The α1 connexin (Cx43 or *Gja1*) is predominantly expressed in lens epithelial cells. We have not characterized the properties of epithelial cells in different α8-S50P mutant mice. It would be an interesting area to explore in future studies.

Fiber cell elongation is a morphological hallmark of lens fiber cell differentiation. The mechanism for fiber cell elongation has never been investigated in vivo due to a lack of proper experimental systems. Current understanding of fiber cell elongation is based on the results obtained from elongation studies of cultured lens epithelial cells in vitro (Beebe et al., 1982; Parmelee and Beebe, 1988) and some descriptive in vivo information (Bassnett, 2005; Kuszak et al., 2004). Several transcriptional factors have been reported to be essential for the formation of lens primary fiber cells (Kim et al., 1999; Ogino and Yasuda, 2000; Wigle et al., 1999). However, mechanistic differences between lens primary and secondary fiber formation are unknown. No previous results have ever demonstrated the involvement of α8 connexin or gap junction channels in the regulation of elongation or formation of lens primary fiber cells. This work provides molecular evidence for regulatory differences between lens primary and secondary fiber cells.

The α8-S50P mutant subunits alone are nonfunctional in the lens. The mutant subunits must rely on the presence of wild-type α8 subunits to inhibit the elongation of primary fiber cells. However, the combination of α8-S50P and wild-type α8 subunits does not disrupt the formation of secondary fiber cells in postnatal lenses. As neither $\alpha8^{-/-}$ knockout nor $\alpha8^{-/-}$ $\alpha3^{-/-}$ double knockout inhibits the elongation of lens primary fibers, a complete blockage of fiber-to-fiber coupling is not responsible for the inhibition of primary fiber cell elongation. Normal fiber-to-fiber coupling does not inhibit primary fiber cell elongation in wild-type lenses. We hypothesize that gap junction channels formed by α8-S50P and wild-type α8 subunits gain their mutant function by increasing channel permeability to facilitate the fiber-to-fiber transport or by transmitting a unique signal that inhibits the elongation of primary fiber cells. Future experiments will be needed to evaluate this hypothesis or an alternative hypothesis that the interaction of α8-S50P and wild-type α8 subunits perturbs a non-junction signaling event to inhibit the elongation of primary fiber cells.

The molecular basis for the proper formation of differentiating secondary fiber cells and the maturation of fiber cells remains largely unknown despite substantial descriptive information that have been published in the last few decades. This work demonstrates that α8-S50P must rely on the presence of endogenous wild-type α3 to disrupt the formation of secondary fiber cells in postnatal lenses. A previous study of the α8-G22R mutation also revealed the importance of the interaction between mutant α8 and wild-type α3 connexins in regulating the formation of secondary fiber cells (Chang et al., 2002). These results suggest that the intercellular gap junctions formed by α8 and α3 connexin subunits modulate the formation of secondary fiber cells in the lens periphery.

In summary, this work reveals that the α8-S50P mutation causes lens cataracts via distinct mechanisms, which are different from those causing cataracts in the $\alpha8^{-/-}$ knockout and α8-G22R mutant mice. More importantly, this study clearly demonstrates that connexin point mutations such as α8-S50P can be used as useful experimental models to investigate fundamental mechanisms during lens development and cataractogenesis in vivo.

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