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Depression of Intraocular Pressure Following Inactivation of Connexin43 in the Nonpigmented Epithelium of the Ciliary Body

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Abstract

Purpose—Conditional inactivation of connexin43 (Cx43) in the pigmented epithelium of the mouse eye results in a reduction in aqueous humor production and complete loss of the vitreous chamber. It was proposed that gap junctions between pigmented and nonpigmented epithelia of the ciliary body are critical for the production of the aqueous humor. To form such junctions, Cx43 in the pigmented epithelium must interact with connexin(s) present in the adjacent cells of the nonpigmented epithelium. The importance of Cx43 expression in the nonpigmented epithelium for the establishment of gap junctions and the regulation of intraocular pressure was tested.

Methods—To inactivate Cx43 in the nonpigmented epithelium of the mouse eye, a mouse line was crossed with a floxed Cx43 locus (Cx43^{flox/flox}) and a transgenic mouse line expressing *cre* recombinase under the control of the Pax6 α promoter. General eye structure was evaluated by light microscopy, gap junctions were analyzed by electron microscopy, and intraocular pressure was directly assessed with micropipettes.

Results—In Pax6 α -*cre*/Cx43^{flox/flox} mice, Cx43 was partially inactivated in the nonpigmented epithelium of the ciliary body and iris. Animals developed dilatations between the pigmented and nonpigmented epithelia and displayed a significant reduction in intraocular pressure. However, gap junctions between the ciliary epithelial layers were decreased but not eliminated.

Conclusions—Cx43 expression in the nonpigmented epithelium of the ciliary body contributes to the formation of gap junctions with the cells of the pigmented epithelium. These gap junctions play a critical role in maintaining the physical integrity of the ciliary body epithelium. Although the partial loss of Cx43 from the nonpigmented epithelium was correlated with a measurable drop in intraocular pressure, possible changes in Cx43 in the aqueous outflow pathway may provide an additional contribution to the observed phenotype.

The aqueous humor provides oxygen and metabolites to the ocular tissues, and the balance between the rates of formation and absorption of the aqueous humor determines intraocular pressure (IOP), which maintains the curvature of the cornea and the refractive properties of the eye. The aqueous humor is produced in the ciliary body¹ by a double-layered epithelium in which apical surfaces of the epithelial cells are opposed. Gap junctions are abundant at these

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opposing surfaces between pigmented epithelia (PE) and nonpigmented epithelia (NPE; heterologous junctions) and are present between adjoining cells in each layer (homologous junctions).² In a current model of ciliary epithelium function, the heterologous junctions join the two epithelia in an ionic syncytium, permitting them to function coordinately to produce and secrete the aqueous humor.³

Gap junctions are composed of intercellular channels that mediate the movement of ions and low-molecular-weight metabolites directly between the cytoplasm of adjacent cells.^{4–6} Intercellular channels are composed of connexins. Twenty mouse genes and 21 human genes for connexins have been identified.⁷ Connexins are differentially expressed, with some present in only a few cell types and others more widespread.^{8–14} Connexin43 (Cx43) is prominently expressed in diverse eye structures such as the ciliary body^{15–17} and in lens and corneal epithelia.^{18–20} Cx43 mutations are associated with a human disease called oculodentodigital dysplasia (ODDD), which is characterized by pleiotropic developmental anomalies of the eyes, face, limbs, and teeth.^{21–24} Although glaucoma is found in some of the families, it is not possible to link changes in intraocular pressure directly with the mutations in Cx43. A mouse model of oculodentodigital dysplasia has been generated in which a mutation of a highly conserved amino acid G60S, in Cx43, leads to some of the symptoms of this disease, including corneal opacities and malformation of the iris.²⁵ A range of phenotypes, however, is consistent with the pleiotropic nature of the clinical syndrome.

Our laboratory has previously found that the deletion of Cx43 in mice causes no morphologic alterations in the prenatal eye.²⁶ However, these animals die at birth as a result of a cardiac malformation.²⁷ To explore the role of Cx43 in post-natal eye development, we produced a mouse line in which Cx43 was selectively inactivated in the PE but not the NPE of the ciliary body. These animals exhibited severe eye abnormalities, including complete loss of the vitreous humor from the posterior chamber.¹⁷ This was a surprising result in that both the PE and the NPE have been reported to express Cx40.¹⁶ If so, Cx40 in the PE of our conditional knockout should have been able to form intercellular channels with either Cx40²⁸ or the remaining Cx43 in the NPE and thus support metabolic coupling between the layers of epithelia.

Together, the data suggest that the production of aqueous humor requires the presence of gap junctions between PE and NPE containing solely Cx43. To test this idea further, we produced a mouse line in which Cx43 was selectively inactivated in the NPE by crossing a floxed Cx43 mouse line to a transgenic line expressing *cre* recombinase under the control of the retina-specific regulatory element α of the murine Pax6 promoter.²⁹ In this study, we compared Pax6 α -cre/Cx43^{+/flox} (referred to as control) with Pax6 α -cre/Cx43^{flox/flox} (referred to as knockout) animals. Transgenic mice display *cre* activity in all retinal progenitor cells in the distal neuroretina and in the iris and ciliary epithelium (which derive from the distal retina) but not in the retinal pigment epithelium, lens, or cornea.³⁰ Knockout mice showed a substantive loss of Cx43 expression in the NPE but not the PE, with a subsequent mild reduction in intraocular pressure. We were unable to confirm the presence of Cx40 in the ciliary epithelium or its potential to rescue the loss of Cx43. Our results confirm that Cx43 plays a key role in gap junction formation between the two epithelia and the production of aqueous humor. The critical functional role of Cx43 in the regulation of IOP suggests a putative pharmacologic target in the treatment of glaucoma, a pathologic state in which high IOP is a well-known risk factor.

Methods

All procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Mice

The generation of the Cx43^{flox/flox} mice has been described previously.³¹ The Pax6 α -cre transgenic line was obtained from Tyler Jacks (Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA). The ROSA26 reporter mouse was provided from Susan Dymecki (Department of Genetics, Harvard Medical School, Boston, MA). Littermate cre-positive, Cx43^{+/flox} heterozygotes were used as controls in all experiments unless otherwise indicated. Mice were housed and cared for according to institutional guideline.

PCR Analysis

Mice were genotyped by PCR.³² Primer sequences were as follows: Cre, 5'-attgctgcattaccgggc-3' and 5'-atcaacgtttcttttcgga-3'; ROSA26-lacZ reporter, 5'-ccgacggcagctgattgaag-3' and 5'-atgcggtcgctgctgggtgc-3'; Cx43 wild-type and floxed (PCR products of 490 and 580 bp, respectively), 5'-cttgactctgattacagagcttaa-3' and 5'-gtctcactgttacttaacagcttga-3'.

β -Galactosidase Histochemistry

To determine the expression pattern of Pax6 α -cre in the mouse eye, Pax6 α -cre/ROSA26 or ROSA26 control littermate mice were anesthetized with 5 to 10 μ L of 100 mg/mL ketamine and perfused intracardially first with Ame's medium (pH 7.4; Sigma, St. Louis, MO) containing 10 U/mL heparin, and subsequently with 4% paraformaldehyde in Sorenson buffer (pH 7.4). Eyes were removed, postfixed for 4 hours in 1% glutaraldehyde, washed three times for 10 minutes with PBS, incubated overnight at 4°C in 20% sucrose in PBS, then frozen in OCT by immersion in liquefied propane/N₂. For histochemical detection of β -galactosidase activity, 14- μ m frozen sections were prepared. Sections were incubated at 37°C with 1 mg/mL X-gal, 2 mM MgCl₂, 0.1% sodium deoxycholate, 0.02% NP-40, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide in phosphate buffer (pH 7.3).³³ Light micrographs were captured with a digital camera (SPOT RT; Diagnostic Instruments, Sterling Heights, MI) mounted on a photomicroscope (E800; Nikon, Tokyo, Japan).

Immunofluorescence

Mice were euthanatized by CO₂ inhalation followed by decapitation. Eyes were fixed by immersion in 4% paraformaldehyde in PBS (pH 7.4) overnight at 4°C, then rinsed in PBS, dehydrated, embedded in paraffin, sectioned (5 μ m), and mounted. For immunostaining, slides were deparaffinized, treated with 10 μ g/mL proteinase K (Sigma) for 5 minutes at 37°C, and blocked in 5% bovine serum, 1% bovine serum albumin, and 0.2% Triton X-100 in PBS (pH 7.4) for 1 hour. Deparaffinized sections were prepared as described previously¹⁷ and were incubated with rabbit polyclonal antibody raised against residues 360 to 382 of rat Cx43 (Sigma). For Cx40 localization, unfixed specimens were directly frozen in liquid propane. Frozen sections were immersed for 5 minutes in methanol at -20°C and then in 5% bovine serum, 1% bovine serum albumin, 0.2% Triton X-100 in PBS (pH 7.4) at 4°C. Sections were subsequently stained with anti-Cx40 antibody (Alpha Diagnostic International, San Antonio, TX). Images were collected with a photomicroscope (E800; Nikon) equipped with a digital camera (SPOT-2; Diagnostic Instruments). Five-megabyte tiff images were collected and contrast optimized with imaging software (Photoshop; Adobe, San Jose, CA). Image collages were assembled and labeled in a graphics suite (CorelDraw; Corel Corporation, Mountain View, CA), then exported as final jpeg files.

Electron Microscopy

Mice were euthanatized by CO₂ inhalation, and the eyes were enucleated. The posterior half of the globe was cut away with scissors in fixative (4% formaldehyde, 2.5% glutaraldehyde, 2% CaCl₂, 0.1 M cacodylate buffer, pH 7.4). The lens was removed by cutting of the zonular

fibers. For immunocytochemical studies, fresh hemisected anterior eyes were fixed in 1% formaldehyde for 30 minutes followed by removal of the lens. The specimens were rinsed three times in 100 mM Tris, 0.85% NaCl (TBS), pH 8.0, and washed for 1 hour in TBS with 0.1% glycine and 0.05% Triton X-100, pH 8.0. Specimens were incubated with anti-Cx43 antibody (diluted 1:1 with 2× TBS, 0.2% BSA, and 0.1% Triton X-100) overnight at 4°C followed by 4 washes, 2 hours each in 1× TBS, 0.1% BSA, and 0.05% Triton X-100. Specimens were then immersed for 1 hour in 5-nm gold-labeled goat anti-rabbit IgG (AuroProbe EM GAR G5; Amersham, Piscataway, NJ). After incubation and 2 washes, 1 hour each in PBS, the specimens were fixed again in 2.5% glutaraldehyde with 1% tannic acid in 100 mM sodium cacodylate, pH 7.4, postfixed in 1% OsO₄, stained in 1% uranyl acetate, and dehydrated in graded ethanol.³⁴ Specimens were embedded in epoxy resins, and 70-nm sections were cut for electron microscopy. Electron microscope sections were studied unstained or after routine lead citrate. All electron micrographs were collected with a transmission electron microscope (1200EX; JEOL, Tokyo, Japan) at 80 kV using conventional and digital plates.

In Situ Hybridization

In situ hybridization was performed as described previously.³⁵ In brief, frozen sections (14 μ m) of adult mice were fixed with 4% paraformaldehyde in DEPC-PBS (pH 7.4) for 10 minutes and treated with 10 μ g/mL proteinase K (Roche, Indianapolis, IN) for 10 minutes at room temperature. Sections were hybridized with a digoxigenin-labeled cRNA probe prepared from cDNA encoding full-length rat Cx43 overnight at 58°C. After hybridization, sections were blocked and incubated for 1 hour with peroxidase-anti-digoxigenin antibody (Roche). Sections were washed, incubated with tyramine-biotin (New England Nuclear, Boston, MA) according to the manufacturer's instructions, and incubated with streptavidin-Alexa 488 (Molecular Probes, Eugene, OR) in the dark for 30 minutes. Sections were mounted and examined with a photomicroscope (E800; Nikon) equipped with a digital camera (SPOT-2; Diagnostic Instruments).

Intraocular Pressure

Nine- to 11-week-old mice were used. Intraocular pressure was determined by using micropipettes with resistance between 0.1 and 0.3 M Ω , as described previously.³⁶ The data are presented as mean $\pm$ (SEM). Statistical significance of differences between values was performed with the one-tailed *t*-test.

Results

Expression Pattern of the Pax6 α -cre Transgene in the Mouse Eye

To inactivate Cx43 in the NPE, we planned to cross Cx43^{flox/flox} mice with a transgenic line expressing *cre* recombinase under the control of Pax6 α promoter, which had been previously reported to express *cre* in the NPE but not the PE.³⁷ To verify *cre* expression in the ciliary processes, we crossed Pax6 α -cre and ROSA26 reporter lines and evaluated β -galactosidase activity, which is observed only after *cre* expression.³⁸ To avoid masking of the signal from β -galactosidase by the melanin abundantly expressed in the PE, we performed these experiments in albino mice. As indicated in Figures 1A and 1B, β -galactosidase activity was strongly detected in the ciliary body and iris. β -Galactosidase activity was observed strongly in the NPE (Fig. 1B, asterisks) but was also observed in the PE (Fig. 1B, arrowheads). β -Galactosidase activity was absent in small areas of the ciliary body (Fig. 1B, arrows). No reaction above background was visible in the corneal angle (angle) where the aqueous outflow pathway is found. The ROSA26 reporter mouse did not display any activity in the absence of *cre* (data not shown). The overall expression pattern of Pax6 α -cre validates our proposal to use the Pax6 α -cre transgenic mouse line to potentially inactivate Cx43 in vivo selectively in the ciliary body.

Reduction of Cx43 Expression by Pax6 α -cre in the Nonpigmented Cells of the Ciliary Body

To test the effectiveness of Cx43 inactivation by Pax6 α -cre at the mRNA level, we performed *in situ* hybridization studies on frozen sections prepared from Pax6 α -cre/Cx43^{+/flox} (control) or homozygous (Pax6 α -cre/Cx43^{flox/flox} (knockout) mice. In the control animals, a strong hybridization signal of the Cx43 probe revealed a high expression level for Cx43 in the ciliary processes. Cx43 mRNA was detected in the NPE of the ciliary epithelium (Fig. 2A). In the knockout animals, signal was dramatically reduced in the NPE (Fig. 2C). Because of the masking effect of the pigment granules, the degree of knockout of signal in the PE was not possible to assess.

To confirm that Pax6 α -cre also reduced Cx43 at the protein level, we performed immunostaining studies with a selective Cx43 antibody on paraffin sections. We demonstrated previously that this antibody showed no staining in the ciliary epithelium from an E15 Cx43^{-/-} mouse.¹⁷ As shown in Figure 3A, controls displayed cytoplasmic signal in the NPE in addition to a strong punctuate signal for Cx43 at the interface of PE and NPE of the ciliary body. In the ciliary body of the knockout mouse, the cytoplasmic staining for Cx43 in the NPE was reduced to background levels (Fig. 3B). In addition, signal was generally absent from the interface between pigmented and nonpigmented cells (open arrow), though some areas still displayed a weak Cx43 signal (Fig. 3B, closed arrow).

Overall Ocular Morphology Unchanged in Knockout Mice

Whole eyes from 18-week-old knockout mice were prepared for light microscopy, and paraffin sections were examined in the light microscope. Figure 4A presents a low-magnification photomicrograph showing cornea, Schlemm's canal, iris, lens ciliary body, distal retina, and uvea. Figure 4B offers a higher magnification view of the ciliary body and retina. There were no observable differences between these specimens and control littermates (data not shown).

Partial Separation of the Pigmented from the Nonpigmented Epithelium as a Result of Cx43 Reduction

The ciliary epithelia were evaluated by electron microscopy to determine whether loss of Cx43 caused morphologic alterations in the ciliary processes and whether gap junctions at the PE/NPE interface were eliminated. The ciliary body of the Cx43-deficient mouse displayed numerous dilatations between the PE and NPE (Figs. 5B, 5D). These spaces were not observed in the controls (Figs. 5A, 5C). A complete separation between the two epithelial layers was prevented by the presence of specialized cell-cell interactions (Fig. 6A, asterisks). Some of these interactions were morphologically similar to adherens junctions (inset, Fig. 6A, double asterisk), whereas others were novel interactions with more closely apposed plasma membranes and electron density in the intercellular space (inset, Fig. 6A, single asterisk). Occasionally, gap junctions were found between PE and NPE cells in the knockout specimens (Fig. 6B, asterisk and inset). In these areas, no dilatations between the two epithelial layers were observed.

Cx43, the Predominant Connexin Forming Gap Junctions in the Ciliary Epithelium

Although we have previously demonstrated that Cx43 expression in the PE is critical for gap junction formation between the PE and the NPE of the ciliary body,¹⁷ the specific connexin family member in the NPE that participates in the formation of these gap junctions is still unknown. To determine the connexin identity in these gap junctions, we evaluated the presence of Cx43 by immunoelectron microscopy. Because the penetration of the gold-labeled secondary antiserum was determined to be inconsistent, we relied on tannic acid staining of the primary antibody.³⁹ With the use of this method, the cytoplasmic surfaces of antibody-labeled junctions were enhanced. In the controls, all gap junctions at the PE/NPE interface

were uniformly labeled (Fig. 7A, arrow) with the primary antibody evident on both junctional cytoplasmic surfaces (inset, Fig. 7A; compare with inset, Fig. 6B). No unlabeled gap junctions were found. In the knockout specimens, the PE/NPE interface (arrows) showed infrequent antibody labeling though the label was reliably detected in the homologous PE/PE gap junctions (Fig. 7B, asterisk). Figure 8 shows that the rare gap junctions at the PE/NPE interface in knockout epithelia also contained Cx43 on both sides. In this case, the specimen was counterstained with a gold-labeled secondary antibody, confirming the identity of the tannic acid-stained material.

Because it has been reported that Cx40 is expressed in the ciliary body of the rat,¹⁶ we tested whether this connexin was also present in the mouse eye. Previously, we found nonspecific Cx40 staining in the ciliary body that was also present in Cx40^{-/-} specimens, making assessment of the presence of Cx40 problematic.¹⁷ We found that this nonspecific staining could be minimized by performing immunostaining on unfixed, frozen sections (Fig. 9). Under these conditions, we were unable to detect any positive Cx40 signal in the ciliary epithelium (Fig. 9A). As a positive control, characteristic junctional staining was present in the endothelial cells from a uveal arteriole in the same section (Fig. 9C, arrows). We conclude that in contrast to the rat, Cx40 does not seem to be involved in mediating intercellular communication through gap junctions in the mouse ciliary epithelium. Taken together, these data indicated that Cx43 comprised homomeric and homotypic gap junctions between the PE and NPE in the mouse and that rescue by Cx40 was not apparent.

Reduction of Intraocular Pressure in the Cx43-Deficient Mouse

To determine whether the reduction of Cx43 expression in the NPE of the ciliary body affected the IOP, we measured the IOP in eyes from 9- to 11-day-old control and knockout (experimental) mice. Inactivation of Cx43 resulted in a significant decrease in the IOP from 15.3 ± 1.5 mm Hg ($n = 10$) in control mice to 11.7 ± 1.2 mm Hg ($n = 10$) in knockout mice. With the one-tailed *t*-test, the probability of the null hypothesis was 0.0385. The data are graphically presented in Figure 10.

Changes in Cx43 Expression in Other Ocular Tissues

Given that Cx43 may be removed from other ocular structures as a result of Pax6 α -cre removal of the floxed Cx43 locus, we examined the distribution of Cx43 in all ocular tissues. As shown in a control specimen (Fig. 11A), Cx43 staining was evident in the ciliary epithelium, corneal epithelium and endothelium, iris, conjunctiva, and retinal pigment epithelium (RPE). The staining in the lens was shown previously to be an artifact.¹⁷ There was also an increased background in the ganglion cell/inner plexiform layer of the retina that, at higher magnification, was diffuse and not organized into junctional maculae. In the knockout animal (Fig. 11B), there were no changes in the staining seen in the corneal epithelium and endothelium or in the conjunctiva and RPE. There were changes in the amounts of staining in the ciliary epithelium, as described in more detail in earlier figures. In addition, there was a diminished level of Cx43 seen in the iris at the level of the posterior PE and the anterior myoepithelium (AME). Because these cellular levels are closely apposed, electron microscopy will be required to assess which cell types have lost Cx43 immunoreactivity. We did not observe miosis in these animals, as was observed in the Nestin-cre mouse line,¹⁷ suggesting that Cx43 may still join the AME cells in the plane of the iris.

Discussion

A current model^{40,41} postulates that gap junctions play a key role in aqueous humor formation. Ions are taken up from the vascularized stroma by transporters located in the basolateral membrane of PE cells, diffuse through gap junctions connecting PE and NPE cells, and are

released to the posterior chamber of the eye. Transport of these ions generates an osmotic gradient that draws water across the ciliary epithelium to produce the aqueous humor. Tight junctions between the NPE cells deny backflow from the aqueous to the stroma, forming part of the blood-aqueous barrier. We had shown previously that inactivation of Cx43 in the PE of the ciliary epithelium using *cre* recombinase expression driven by the nestin promoter results in a substantive loss of intraocular pressure (IOP) leading to severe ocular abnormality.¹⁷ The reduced IOP is lower than venous pressure in the canal of Schlemm, resulting in back-diffusion of plasma proteins into the aqueous compartment. It was not known, however, whether the NPE contributed Cx43 to the PE/NPE junctions. Because Cx40 had been reported to form gap junctions at the PE/NPE interface, it is possible that Cx40/Cx40 homotypic gap junctions could still function in interepithelial transport or that Cx43/Cx40 heterotypic junctions were active.

We have used the Pax6 α promoter to drive *cre* recombinase expression in the mouse and have confirmed expression in the anterior eye, particularly the ciliary epithelium. These animals were crossed with a Cx43^{flox/flox} mouse line. In situ hybridization and light and electron microscopic immunocytochemistry revealed that the Cx43 was substantively reduced in the NPE in these mice, though regions of Cx43 expression persisted. Nonetheless, the loss of Cx43 was sufficient to significantly reduce IOP in these animals. It has been shown that the Pax6 α element is active during embryogenesis,²⁹ long before we are able to see any changes in Cx43 expression in the eye or, importantly, any changes in the IOP. Thus, we are not able to make any clear temporal correlation between the loss of Cx43 in the ciliary epithelium and the measured decreases in IOP.

The data between the two knockout lines are difficult to reconcile for technical reasons. Micropipette measurements can only be made on 9- to 11-week-old animals. This is possible with the Pax6 α -*cre* line (present study) but not feasible with the nestin-*cre* line¹⁷ because the nestin-*cre* eyes are almost completely collapsed by that age and have almost no experimentally available aqueous humor. Nestin-*cre* animals begin to show plasma proteins in the aqueous humor by 3 weeks of age, implying that the IOP has fallen below venous pressure in the canal of Schlemm, estimated at approximately 4 mm Hg. The lowest outlier in the Pax6 α -*cre* line is above 5 mm Hg at 7 to 9 weeks (Fig. 10); hence, it is clear that nestin-*cre* animals experience a substantially larger loss in IOP than the Pax6 α -*cre* line.

We propose that the remaining Cx43-containing gap junctions supply a sufficient pathway across the ciliary epithelium such that the IOP is reduced but not critically eliminated in the Pax6 α -*cre* line. It is possible that the IOP changes observed in both mouse models also depended on changes in Cx43 expression by cells—the endothelial cells lining the trabecular mesh-work and the canal of Schlemm—in the aqueous outflow pathway. Cx43 has been immunolocalized in the trabecular meshwork cells.⁴² These cells have an extremely thin morphology; the lack of detection of a positive *cre*-recombinase signal (Fig. 1) and positive Cx43 immunohistochemistry (data not shown) could well have resulted from technical limitations. Thus, though our data are consistent with a depression in cell-cell communication between PE and NPE layers of the ciliary epithelium underlying the change in IOP, important changes in the outflow pathway may provide an alternative explanation.

Through immunocytochemistry, we found all gap junctions at the PE/NPE interface to contain Cx43 in both plasma membranes; even those junctions remaining after Pax6 α -*cre* induced the deletion of Cx43. According to previous reports in the rat, Cx40 is expressed in the ciliary body at the PE/NPE interface.^{15,16} However, we did not detect Cx40 in the mouse ciliary epithelium under conditions in which a clear signal was evident between vascular endothelial cells on the same sections. Thus, we conclude that Cx43 is the primary ion-conducting pathway between the PE and the NPE and that this connexin is required for aqueous humor secretion, though we cannot exclude the possibility that additional connexins may play an ancillary role. This

conclusion is consistent with a recent observation presented in preliminary form that partial knockdown of Cx43 inhibits conductance and dye transfer across gap junctions linking fresh bovine NPE/PE cell couplets (Do CW, et al. *IOVS* 2008;49: ARVO E-Abstract 2103).

After Cx43 inactivation in the NPE, we observed dilatations formed between cells no longer able to assemble gap junctions. It is possible that these dilatations result from interrupted or misplaced transport functions of the NPE cells, resulting in fluid accumulation in the extracellular space. The dilatations were flanked by adherens-type junctions and a novel cell-cell junction that was morphologically distinct. Examination of the PE/NPE interface in Cx43^{-/-} prenatal embryos at embryonic days 15 to 18 revealed similar cell-cell interactions (data not shown), suggesting that they may be related to a step in the assembly process of the gap junctions in advance of connexin recruitment, analogous to the “formation plaques” described in freeze-fracture replicas of assembling gap junctions in Novikoff hepatoma cells.

43

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Figure 1.

Pax6 α -cre promoter drives cre recombinase expression in the ciliary epithelium and iris. (A) ROSA26 mice were mated with *Pax6* α -cre mice and anterior eyes sectioned and histochemically probed for active β -galactosidase. Reaction product is visible in the ciliary body (CB) and in the anterior myoepithelium and posterior pigment epithelium of the iris. No reaction above background is visible in the corneal angle (angle) where the aqueous outflow pathway is found. (B) Higher magnification of the ciliary body demonstrates β -galactosidase reaction product in both the NPE (*asterisks*) and at lower levels in the PE (*arrowheads*) of the ciliary epithelium, though occasional gaps in staining are evident (*arrows*).

Figure 2.

In situ hybridization (**A**, **C**) with Cx43-specific probe reveals reduction of Cx43 mRNA in the ciliary epithelium (CE) of the ciliary body with fluorescent probe. (**B**, **D**) Parallel DIC images of sections. Cx43 mRNA is seen in the CE in the control animal (**A**) that is lacking in the knockout specimen (**C**).

Figure 3.

Immunofluorescent localization of Cx43 protein in ciliary epithelium. **(A)** In the control animal, anti-Cx43 antibody stains cytoplasmic structures in the NPE and is seen localized in discrete maculae at the NPE/PE interface (*arrows*) in addition to intracellular staining. **(B)** In the knockout specimen, the Cx43 staining in the NPE is substantively reduced but still evident in the PE. In most areas, there is little detectable anti-Cx43 staining at the NPE/PE interface (*open arrow*), although in other areas, staining is evident (*closed arrow*).

Figure 4.

Hematoxylin and eosin histology reveals that the overall structure of the anterior eye is not changed in the knockout animals. The cornea, iris, Schlemm's canal, ciliary body, lens, retina, and uvea are normally developed in this 18-week-old eye. (A) Low magnification of anterior eye; (B) high magnification of ciliary epithelium.

Figure 5.

Cx43 inactivation with Pax6 α -cre is associated with dilatations between NPE and PE layers of the ciliary body. Electron microscopy of the NPE/PE revealed regions of clear separation between the NPE layers in the knockout specimen (B) that were not visible in the controls (A). AqH, aqueous humor space in the posterior chamber. (C) At higher magnification, the PE and NPE layers in the control are joined by gap junctions (*asterisk*), and tight junctions (TJ) join adjacent NPE cells. (D) In the knockout, dilatations (*asterisks*) are seen separating cell-cell attachments between the PE and NPE cells.

Figure 6.

(A) Dilatations in the NPE-PE interface of the ciliary epithelium in knockout mice are interrupted by cell-cell interaction structures. These structures appear to contain both adherens junctions (*inset, double asterisk*) and a close apposition that is novel to the loss of Cx43 (*inset, single asterisk*) because it is not seen in the control. (B) Consistent with the appearance of anti-Cx43 staining by immunohistochemistry (see Fig. 3), it is possible to find cell pairs in which gap junctions continue to join the NPE and PE (*asterisk*). In these areas, no dilatations are found. *Inset*: gap junction at the NPE/PE interface at higher magnification in the knockout. TJ, tight junction; AqH, aqueous humor space.

Figure 7.

Immunoelectron microscopy of Cx43 in the ciliary epithelium. **(A)** After detergent permeation, the primary antibody decorates the cytoplasmic surfaces of gap junctions at the PE/NPE interface in the control ciliary body (*arrow*). The antibody appears as a dense coating on the cytoplasmic surfaces (*inset*, *ab*). **(B)** In the knockout epithelium, the antibody fails to stain any cell-cell interactions at the PE/NPE interface (*arrows*), though gap junctions between PE cells are labeled (*asterisk*).

Figure 8. Infrequent gap junctions between the PE and NPE cells stain with anti-Cx43 antibody (*arrow*). *Inset:* higher magnification of a gap junction at the PE/NPE interface stained with anti-Cx43 and gold-labeled anti-rabbit secondary reagent.

Figure 9.

Immunofluorescent localization of Cx40 in the mouse eye. Whole, unfixed, frozen sections of eyes were stained with an anti-Cx40 antibody. (A) Immunofluorescent image of ciliary body showing no staining of the ciliary epithelium. (B) Companion DIC image indicating location of the NPE. (C) A positive control for the anti-Cx40 reagent can be found in the same section by locating a uveal arteriole. The positive inter-endothelial gap junction staining (*arrows*) is visible adjacent to the internal elastic membrane (*iem*).

Figure 10.

Effect of conditional inactivation of Cx43 on mouse IOP. The horizontal lines within the boxes are the medians for the experimental ($n = 10$) and control ($n = 10$) animals. The *lower* and *upper borders* of the boxes present the 25th and 75th percentiles, and the *whiskers* represent the 10th and 90th percentiles. Outlying data are entered beyond the whiskers.

Figure 11. Low-magnification immunofluorescence images of control (**A**) and knockout (**B**) sections of anterior eye stained with anti-Cx43 antibody. Decreased staining is seen in the ciliary epithelium and iris, with no detectable changes in Cx43 expression in other ocular tissues.