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

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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 (Cx43flox/flox) and a transgenic mouse line expressing cre recombinase under the control of the Pax6 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-cre/Cx43flox/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. (Invest Ophthalmol Vis Sci. 2009;50:2185–2193) DOI:10.1167/iovs.08-2962

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vated 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. In this study, we compared Pax6cre/Cx43floxed/lox (referred to as control) with Pax6cre/Cx43floxed/lox (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. 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 Cx43floxed/lox mice has been described previously. The Pax6cre 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, Cx43floxed/lox homozygotes were used as controls in all experiments unless otherwise indicated. Mice were housed and cared for according to institutional guidelines.

PCR Analysis

Mice were genotyped by PCR. Primer sequences were as follows: Cre, 5′-attggtgccttctctctgca-3′ and 5′-atacagttccctctctgca-3′; ROSA26lacZ reporter, 5′-cagcagcagctgtaa-3′ and 5′-atcagcagctgtaa-3′; Cx43 wild-type and floxed (PCR products of 490 and 580 bp, respectively), 5′-ctttgactctgattacagagcttaa-3′ and 5′-gtctcagcttctactaa-3′.

β-Galactosidase Histochemistry

β-Galactosidase histochemistry was performed as described previously. Mice were euthanized 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 hemisectioned 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. Specimens were embedded in epoxy resin, and 70-nm sections were cut for electron microscopy. Electron microscopy 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. In brief, frozen sections (14 μm) of adult mice were fixed with 4% paraformaldehyde in DEPC-PBS (pH 7.4) for 10 minutes and treated with 10 μg/mL protease 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). Electron microscopy 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.

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 MΩ, as described previously. The data are presented as mean ± SEM. Statistical significance of differences between values was performed with the one-tailed t-test.
RESULTS

Expression Pattern of the Pax6α-cre Transgene in the Mouse Eye

To inactivate Cx43 in the NPE, we planned to cross Cx43flx/flx mice with a transgenic line expressing cre recombinase under the control of Pax6α 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 Pax6α-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 Pax6α-cre validates our proposal to use the Pax6α-cre transgenic mouse line to potentially inactivate Cx43 in vivo selectively in the ciliary body.

Reduction of Cx43 Expression by Pax6α-cre in the Nonpigmented Cells of the Ciliary Body

To test the effectiveness of Cx43 inactivation by Pax6α-cre at the mRNA level, we performed in situ hybridization studies on frozen sections prepared from Pax6α-cre/Cx43flx/flx (control) or homozygous (Pax6α-cre/Cx43flx/flx) (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 Pax6α-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 Cx43flx/flx 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, the specific connexin family member in the NPE that participates in the formation of these gap junctions is still unknown. To determine the con-

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**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 substantially 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.
nexit 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. 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, 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. 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-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 endo-
The staining in the lens was shown previously to be an artifact. 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

**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.
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were crossed with a Cx43flox/flox mouse line. In situ hybridization showed that the IOP has fallen below venous pressure in the canal of Schlemm, estimated at approximately 4 mm Hg. The lowest outlier in the Pax6α-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 Pax6α-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 Pax6α-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 meshwork and the canal of Schlemm—in the aqueous outflow pathway. Cx43 has been immunolocalized in the trabecular meshwork cells. 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.

**DISCUSSION**

A current model postulates that gap junctions play a key role in aqueous humor formation. Ions are taken up from 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. 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 Pax6α 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 Cx43flox/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 Pax6α element is active during embryogenesis, 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 Pax6α-cre line (present study) but not feasible with the nestin-cre line 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 Pax6α-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 Pax6α-cre line.
Through immunocytochemistry, we found all gap junctions at the PE/NPE interface to contain Cx43 in both plasma membranes; even those junctions remaining after Pax6a-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 dilations formed between cells no longer able to assemble gap junctions. It is possible that these dilations result from interrupted or misplaced transport functions of the NPE cells, resulting in fluid accumulation in the extracellular space. The dilations 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.45

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References


