

Optimal Lens Epithelial Cell Proliferation Is Dependent on the Connexin Isoform Providing Gap Junctional Coupling

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PURPOSE. Gap junctions between epithelial cells are essential for normal lens growth. In mice, knockout of Cx50 or targeted replacement of Cx50 with Cx46 (knockin) caused smaller lenses because of decreased epithelial cell proliferation. However, it remains unclear whether Cx50 functionally contributes to lens epithelial coupling during maximal proliferation on postnatal day 2 (P2) and P3. To determine which connexins functionally contribute to epithelial cell coupling and proliferation, junctional coupling from epithelial cells of wild-type and knockin mice was examined.

METHODS. Epithelial cells were isolated from wild-type or knockin mice at different developmental ages. Junctional currents were measured by dual whole cell voltage clamp. Cell proliferation was assayed by BrdU incorporation. Connexins were immunolocalized using specific antibodies.

RESULTS. Junctional currents between lens epithelial cells exhibited a developmentally regulated sensitivity to quinine, a drug that blocks Cx50 gap junctions, but not Cx43 or Cx46. Single-channel currents had a unitary conductance of 210 pS, typical of Cx50. Immunocytochemical staining showed Cx43 and Cx50 were abundantly expressed in wild-type cells, and Cx46 replaced Cx50 in knockin cells. A correlation between functional activity of Cx50 and maximal proliferation was also found. In epithelial cells from P3 wild-type mice, there was a high density of BrdU-labeled nuclei in both the central epithelium and the equatorial epithelium, and 60% or more of total coupling was provided by Cx50. In older cells, proliferation was greatly reduced, and the contribution of Cx50 to total coupling was progressively reduced (45% or less on P12; 25% or less on P28). Coupling between epithelial cells of Cx46 knockin mice was similar in magnitude to that of wild-type mice but had pharmacologic and biophysical characteristics of Cx46. This functional replacement of Cx50 with Cx46 was correlated with 71% and 13% reductions in BrdU-labeled cells in the P3 central epithelium and equatorial epithelium, respectively.

CONCLUSIONS. These results reconcile previous genetic studies showing that Cx50 influences epithelial cell proliferation, with numerous studies suggesting that Cx43 was the principal epi-

thelial cell connexin. They further show that the contribution of Cx50 is highest during peak postnatal proliferation but progressively declines with age thereafter. (*Invest Ophthalmol Vis Sci.* 2007;48:5630–5637) DOI:10.1167/iovs.06-1540

Intercellular communication through gap junctions plays a vital role in diverse cellular processes throughout development,¹ such as influencing decisions of cell fate (eg, extent of proliferation or differentiation).^{2,3} Historically, gap junctional communication has been linked to the suppression of aberrant growth in pathologic conditions such as cancer.^{4–7} In contrast, in tissues such as ocular lens, gap junctional communication was found to be necessary for normal growth.^{8–10}

The lens contains a monolayer of epithelial cells at the anterior surface and a mass of elongated fiber cells that extend from the anterior to the posterior sutures. Epithelial cells proliferate, migrate to the equator, and differentiate to produce new fiber cells, leading to radially symmetric lens growth.^{11–13} In the rodent lens, the most rapid growth takes place during the first postnatal week, resulting in increases in lens weight of up to 87% in a single day.¹⁴ Many factors influence the rapid growth of the lens in the first postnatal week, including connexin-mediated intercellular communication between epithelial cells.

The lens expresses three connexins—Cx43, Cx46, and Cx50^{15–17}—with distinct pharmacologic and electrophysiological properties. Cx50 forms large conductance gap junction channels (approximately 220 pS), whereas Cx43 and Cx46 form gap junctions with unitary conductance of 100 pS and 140 pS, respectively.¹⁸ In addition to these differences in single-channel behavior, the three connexins are differentially affected by a variety of pharmacologic agents, including anti-malarial drugs such as quinine and mefloquine, which preferentially block Cx50 but not Cx43 or Cx46.^{19,20}

In mammalian lenses, Cx43 is present only in epithelial cells, whereas Cx46 is absent from epithelial cells but becomes expressed during fiber differentiation. Cx50 is abundantly expressed in epithelium and lens fiber cells.²¹ Previous studies on genetically engineered mice have identified an obligatory role for Cx50 in lens growth and epithelial cell proliferation. Knockout of Cx50 produced small lenses, with growth failure evident during the first postnatal week.^{8,9} In addition, replacement of Cx50 with Cx46 by genetic knockin was insufficient to restore growth.¹⁰ Epithelial cell proliferation was strongly reduced in Cx50 knockout mice on postnatal day (P)2 and P3, when wild-type lenses undergo a large increase in proliferation. Knockin lenses also exhibited decreased cell proliferation on P2 and P3.³ These reductions in the number of dividing cells correlated temporally with the lens mass decreases that were then maintained throughout life. Taken together, these data suggested a specific mitogenic role for Cx50-mediated gap junctional communication.

Although these studies have identified an obligatory role for Cx50 in the promotion of normal lens growth, to date no studies have shown that Cx50 functionally contributes to gap junctional communication between lens epithelial cells. Previ-

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ous electrophysiological studies on cultured mammalian epithelial cells have only characterized gap junctions whose channel properties resemble those formed by Cx43 in exogenous expression systems.^{22,23} Thus, there is an apparent contradiction between genetic studies showing that Cx50 profoundly influences epithelial proliferation and electrophysiological studies, suggesting that Cx50 is not functionally active in epithelial cells.

We have examined the electrophysiological properties of gap junctional communication between epithelial cells during the early postnatal period and adulthood in wild-type and Cx46 knockin mice. We show that wild-type epithelial cells express Cx43 and Cx50, whereas knockin cells express Cx43 and Cx46. We further demonstrate that Cx50 provides most of the functional coupling between epithelial cells during the first postnatal week and is progressively decreased in older animals. This peak of Cx50 functional activity coincided with an increase in the number of mitotic epithelial cells in the first postnatal week, particularly in the central epithelium. In contrast, gap junctional properties between knockin epithelial cells had pharmacologic and electrophysiological characteristics resembling Cx46, and this type of gap junctional communication failed to fully promote postnatal lens growth with a significant reduction of mitotic cells in the central epithelium. These results reconcile the previous genetic studies showing that Cx50 influences epithelial cell proliferation with the electrophysiological studies, suggesting that Cx43 was the principal epithelial cell connexin. They also provide a framework for future studies investigating the precise mechanism whereby Cx50 facilitates lens growth.

METHODS

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

Epithelial Cell Isolation

Lenses were dissected from eyes and transferred to calcium- and magnesium-free phosphate-buffered saline (PBS). The lens capsule was then peeled away from the fiber cell mass using fine forceps. Epithelial cells were dissociated by incubation with 0.25% trypsin for 5 to 7 minutes at 37°C and were plated onto glass coverslips for electrophysiological studies. Junctional currents were typically measured 1 to 4 hours after dissociation, with some exceptions.

Immunocytochemistry

Lenses were dissected, and the lens capsule was peeled away from the fiber cell mass with the use of fine forceps and were pinned down on an encapsulant (Sylgard; Dow Corning, Midland, MI)-coated 35-mm Petri dish.²⁴ Capsules were fixed for 30 minutes with 1% paraformaldehyde in PBS and blocked with 5% BSA in PBS with 0.1% Tx-100 and 0.02% NaN₃ for 1 hour. Epithelial cell gap junctions were stained with polyclonal antibodies to Cx43, Cx46, or Cx50 followed by incubation with a Cy3-conjugated fluorescent goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Cell nuclei were counterstained with DAPI, and capsules were viewed and photographed on a microscope (BX51; Olympus, Tokyo, Japan) using a digital camera (MagnaFire; Optronics, Goleta, CA).

BrdU Injection

P3 or P12 mouse pups were injected intraperitoneally with 100 μ g/g body weight of 5'-bromo-2'-deoxyuridine (BrdU; Sigma, St. Louis, MO). BrdU at 10 mg/mL was dissolved in PBS at 37°C just before use. Injected pups were returned to their mothers. After 1-hour exposure to BrdU, pups were humanely killed, and lenses were dissected. The lens capsule was then peeled away from the fiber cell mass using fine forceps and pinned down on an encapsulant (Sylgard; Dow Corning)-coated 35-mm Petri dish and fixed for 30 minutes in 2% formaldehyde,

made fresh from paraformaldehyde in PBS. Fixed capsules were rinsed with PBS, incubated in 100% MeOH at -20°C for 5 minutes, mounted on microscope slides, and allowed to air dry. BrdU incorporation was immunolabeled with a BrdU in situ detection kit (BD PharMingen, San Diego, CA) according to the manufacturer's instructions, with the exception that endogenous peroxidase was quenched with 0.3% hydrogen peroxide diluted in absolute methanol, and all antibody incubations were carried at 37°C. BrdU-negative nuclei were counterstained with aqueous hematoxylin. Stained sections were viewed with 10 \times and 20 \times objectives on a microscope (BX51; Olympus) and photographed with a digital camera (MagnaFire; Optronics).

Quantitation of BrdU-Labeled Cells

BrdU-positive nuclei were counted in 250 \times 250- μ m square regions of the equatorial or central epithelium from capsules on P3 or P12. The density of labeled cells was calculated for each region of each capsule, and mean density was plotted. Data were collected from four to five capsules on each postnatal day for each genotype. Statistical analyses between wild-type and knockin lenses were performed using the paired Student's *t*-test, with *P* = 0.05 considered significant.

Electrophysiology

Junctional conductance was measured between cell pairs using the dual whole cell voltage clamp technique with 1D patch-clamp amplifiers (Axopatch; Axon Instruments, Foster City, CA) at room temperature. The solution bathing the cells contained 135 mM NaCl, 5 mM KCl, 2 mM CsCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, 5 mM dextrose, 2 mM pyruvate, and 1 mM BaCl₂, pH 7.4. Patch electrodes had resistances of 3 M Ω to 5 M Ω when filled with internal solution containing 125 mM CsCl, 10 mM EGTA, 0.5 mM CaCl₂, and 10 mM HEPES, pH 7.2. Macroscopic and single-channel recordings were filtered at 0.2 to 0.5 kHz and were sampled at 1 to 2 kHz. Data were acquired with appropriate software (PCLAMP8; Axon Instruments) and were analyzed (PCLAMP8 [Axon Instruments] and ORIGIN 6.0 [Microcal Software, Northampton, MA]).

Each cell of a pair was initially held at a common holding potential of 0 mV. To evaluate junctional coupling, 200-ms hyperpolarizing pulses from the holding potential of 0 mV to -20 mV were applied to one cell to establish a transjunctional voltage gradient (V_j), and junctional current was measured in the second cell (held at 0 mV).

The magnitude of coupling between lens epithelial cells was typically high; in some cell pairs, however, the junctional current exhibited a slow run-down that allowed us to resolve unitary currents without the use of uncoupling agents. In addition, the magnitude of coupling was found to decrease when cells were cultured in the absence of serum for more than 12 hours. In such cells, unitary currents were more prevalent. Such single-channel events were recognized as simultaneously occurring events of equal amplitude and opposite polarity in current traces for both cells in the pair.

Although many drugs block Cx50 selectively,^{20,25,26} we preferred to use quinine because its effects are fully and rapidly reversible. For example, quinine strongly reduces Cx50 junctional currents by 90% but has no effect on Cx46 or Cx43 junctional currents. Quinine was obtained from Sigma and was dissolved in the extracellular solution to make a 10-mM stock solution. Solutions were applied with a gravity-fed perfusion system, and solution exchange was complete within 10 seconds. The magnitude of inhibition caused by quinine is expressed as the fraction of the conductance in the absence and presence of the drug. Statistical analyses were performed using the paired Student's *t*-test, with *P* = 0.05 considered significant.

RESULTS

Cx50 Promotes Optimal Cell Proliferation in Central Epithelial Cells

We have previously shown that the loss of Cx50 resulted in a 50% reduction in lens mass and that this growth deficit was

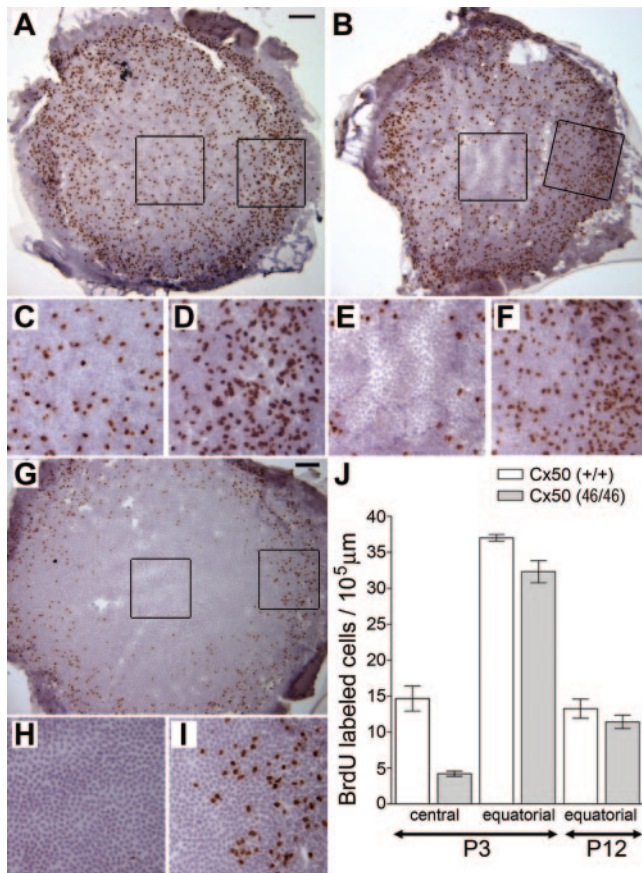


FIGURE 1. Cx50 is required for the proliferation of epithelial cells. Differences in the BrdU incorporation pattern in actively dividing cells in P3 (A–F) and P12 (G–I) lens capsules from wild-type (A, C, D) and Cx46 knockin mice (B, E–I). In wild-type lenses, there were many more BrdU-positive nuclei on P3 (A), and the actively dividing cells could be found in the central (C) and the equatorial (D) epithelium. In Cx46 knockin P3 lenses, there were fewer dividing cells (B), and this reduction was more striking in the central (E) than the equatorial (F) epithelium. By P12, there were fewer dividing cells in knockin (G) and wild-type (not shown) lenses. Labeled cells were absent from the central epithelium (H) and were found only in the equatorial zone (I). Quantification of the BrdU labeling density (J) showed a significant reduction in labeled nuclei in Cx46 knockin lenses in both zones on P3 ($n = 6$) but not on P12 ($n = 5-6$). Data are mean \pm SD. Scale bar, 100 μ m. Boxed areas in A, B, G are enlarged in C–F, H, I.

caused by a sharp reduction in epithelial cell proliferation during the first postnatal week.^{3,8} We have also shown that replacement of Cx50 with Cx46 by homologous recombination (Cx46 knockin) did not rescue the growth defect.^{3,10} Figure 1 illustrates differences in BrdU incorporation after 1-hour exposure in actively dividing cells in P3 and P12 lenses from wild-type or Cx46 knockin mice. On P3, there were many more labeled cells in wild-type than knockin lenses, particularly in the central region of the epithelium. On P12, wild-type and knockin lenses had significantly reduced numbers of BrdU-labeled cells than on P3. In addition, labeled cells were absent from the central epithelium in both cases on P12, and the density of BrdU labeling in the equatorial cells appeared equivalent. Quantitation of the density of BrdU-positive nuclei showed that replacement of Cx50 with Cx46 produced 71% and 13% reductions in labeled cells in P3 central and equatorial epithelium, respectively; these differences were statistically significant ($P < 0.05$). In contrast, by P12, the density of labeled cells in the equatorial epithelium was reduced 65% or more for wild-type and knockin lenses compared with P3. In

addition, though wild-type lenses had a slightly higher density of labeling than knockin lenses on P12, the difference was no longer statistically significant ($P > 0.05$). The dramatic alteration in the number and spatial location of proliferating cells in P3 knockin lenses resulted from genetic manipulation of their gap junction subunits and might have reflected an underlying change in their functional coupling from channel properties resembling Cx50 to those resembling Cx46.

Connexin Expression in Epithelial Cells Changes with Genetic Manipulation

Previous studies have suggested that Cx50 is normally expressed in lens epithelial gap junctions.^{9,27,28} However, high expression levels of Cx50 in lens fibers can make it difficult to unambiguously localize Cx50 in epithelial cell junctions in whole mouse lens sections. To avert this potential problem, we separated the lens capsule with adherent epithelial cells from the underlying fiber cell mass and stained these whole mount preparations with antibodies to the lens connexins. Figure 2 shows staining for Cx43, Cx46, and Cx50 in wild-type and Cx46 knockin epithelial cells. Wild-type lenses expressed Cx43 and Cx50 in punctuate patterns at appositional sites between epithelial cells, whereas Cx46 staining was not detected in wild-type capsules. In Cx46 knockin lenses, Cx43 continued to be expressed at cell–cell interfaces, but Cx50 was no longer detected. In addition, strong punctuate Cx46 staining was now observed, consistent with the genetic replacement of Cx50 by Cx46. These results show that Cx50 is abundantly expressed in wild-type lens epithelial cells and is efficiently replaced by Cx46 in the knockin lenses, but they do not establish whether these changes in connexin subunit expression lead to alteration in properties of gap junctions between epithelial cells.

Magnitude of Coupling Is Similar in Wild-Type and Cx46 Knockin Epithelial Cells

To determine whether the replacement of Cx46 with Cx50 leads to changes in the level of coupling between epithelial cells, we measured the macroscopic coupling in freshly dissociated epithelial cell pairs from lenses of wild-type and knockin mice. We initially chose to measure coupling from lenses of P3 mice because the difference in BrdU incorporation between wild-type and knockin animals was maximal during this time.³

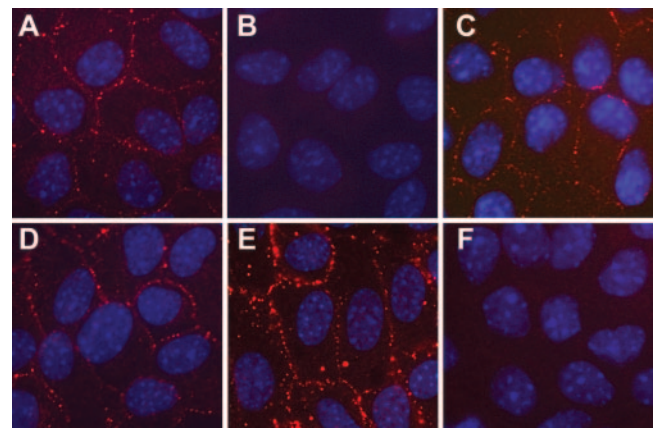


FIGURE 2. Immunofluorescence of the three lens connexins in epithelial cell capsules. Epithelial cell capsules from wild-type (A–C) and Cx46 knockin (D–F) lenses were immunostained with antibodies raised against Cx43 (A, D), Cx46 (B, E), and Cx50 (C, F) and were examined by fluorescence microscopy. Merged images show Cy3 staining of connexins (red) and DAPI staining of cell nuclei (blue). Scale bar, 10 μ m.

The magnitude of coupling on P3 was similar in wild-type and Cx46 knockin lenses (Fig. 3). The coupling conductance ranged from 0.5 nS to 30 nS in wild-type and knockin epithelial cells, and the mean values were not significantly different ($P > 0.05$). In addition, the extent of coupling did not change with age. Coupling levels between epithelial cells from adult lenses of wild-type and Cx46 knockin mice were also similar in magnitude (data not shown). These results indicate that differences in proliferation between wild-type and Cx46 knockin epithelia were not caused by changes in the total levels of coupling between these cells.

Pharmacologic Properties of Gap Junctions Change with Genetic Manipulation

In exogenous expression systems, the three lens connexins respond differently to certain drugs. For example, the antimalarial drug quinine selectively blocks gap junctions formed by Cx50.²⁰ Quinine (300 μ M) typically reduces, by approximately 90%, junctional currents in N2A cells exogenously transfected with Cx50. In comparison, the drug has no effect on currents in Cx43- or Cx46-transfected cells (Fig. 4D for summary of results). Therefore, we determined the effects of quinine (300 μ M) on coupling between epithelial cells isolated from wild-type and knockin lenses (Fig. 4). The effect of quinine on junctional currents from a cell pair obtained from P3 wild-type lenses is shown in Figure 4A. Quinine strongly reduced junctional currents in wild-type epithelial cells, an effect that was rapidly reversible on washout of the drug. The quinine-induced reduction in junctional currents was observed in more than 95% of the cell pairs that were tested and typically averaged 60% to 70% of the initial conductance ($63\% \pm 5\%$; $n = 14$). These results indicated that Cx50 provided a large fraction of the coupling between epithelial cells during the first postnatal week. The quinine-insensitive fraction (approximately 37%) was most likely attributed to Cx43 gap junctions.

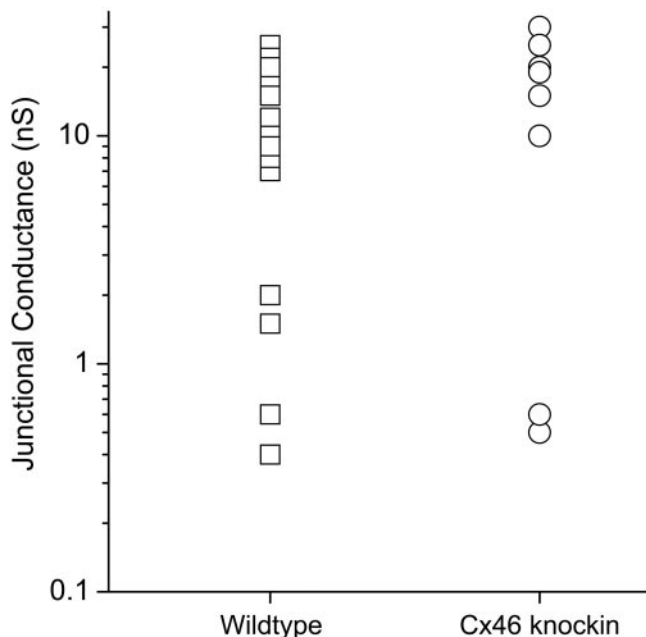


FIGURE 3. The magnitude of gap junctional coupling was similar in wild-type and Cx46 knockin epithelial cells. Junctional conductance was measured using the dual whole-cell patch clamp on epithelial cells dissociated from wild-type and Cx46 knockin lenses. Coupling was measured 2 to 6 hours after dissociation of cells. Epithelial cells from wild-type and knockin lenses had a mean conductance of 12.3 ± 1.5 nS ($n = 27$) and 15.1 ± 3.0 nS ($n = 10$), respectively.

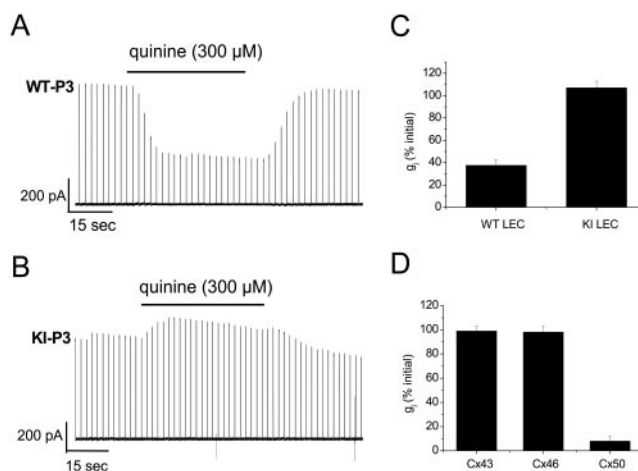


FIGURE 4. Pharmacologic properties of gap junctions in epithelial cells from wild-type and Cx46 knockin lenses. Effect of quinine (300 μ M) on junctional currents in wild-type (A) and Cx46 knockin (B) lenses. Junctional currents were recorded in response to 200-ms pulses to -10 mV applied to one cell of a pair every 2 seconds from a holding potential of 0 mV. Application of quinine strongly and reversibly decreased junctional currents in epithelial cells from wild-type lenses. In contrast, quinine caused a small (approximately 5%) increase in coupling in epithelial cells from knockin lenses. (C) Bar graph summarizing the effect of quinine on coupling in wild-type and Cx46 knockin epithelial cells. Each bar represents the mean \pm SEM of 4 to 17 cell pairs. (D) Quinine selectively blocks Cx50 gap junctions in N2A cells. Bar graph illustrating the selective block of Cx50 gap junctions by quinine. Quinine (300 μ M) has no significant effect on junctional currents in gap junctions in N2A cells exogenously transfected with Cx43 and Cx46. However, this concentration of quinine significantly inhibited Cx50 channels.

In contrast, junctional conductance between epithelial cells from knockin lenses was not reduced by quinine (Fig. 4B). Quinine typically produced a small, transient increase (approximately 5%) in junctional current between knockin cells, though this was not maintained for the entire duration of quinine application and did not achieve statistical significance. The average junctional conductance between knockin cells treated with quinine was $107\% \pm 6\%$ of the initial value ($n = 4$). The differences in sensitivity of wild-type and knockin epithelial cells to quinine are summarized in Figure 4C. These results indicate that the large differences in cell proliferation between wild-type and knockin lens epithelial cells were correlated with the replacement of quinine-sensitive Cx50 gap junctions with quinine-insensitive Cx46 channels.

Single-Channel Properties Differ between Wild-Type and Knockin Epithelial Cells

Previous biophysical studies have documented that Cx43, Cx46, and Cx50 form gap junctions with distinct single-channel conductances.^{19,29,30} Cx50 forms large conductance gap junction channels (200 pS), whereas Cx43 and Cx46 form smaller channels with unitary conductances of 100 pS and 140 pS, respectively. To determine whether large conductance channels corresponding to Cx50 were present in the P3 wild-type epithelial cells, unitary currents were measured in poorly coupled cell pairs. Single-channel currents from two different cell pairs are depicted in Figure 5A. At a transjunctional voltage gradient of -60 mV, single-channel currents were large and frequently exhibited transitions between open, closed, and subconductance states. Amplitude histograms of the recording, illustrated on the right of the trace, showed two distinct peaks at 11.6 pA and 0 pA, corresponding to the fully open state and

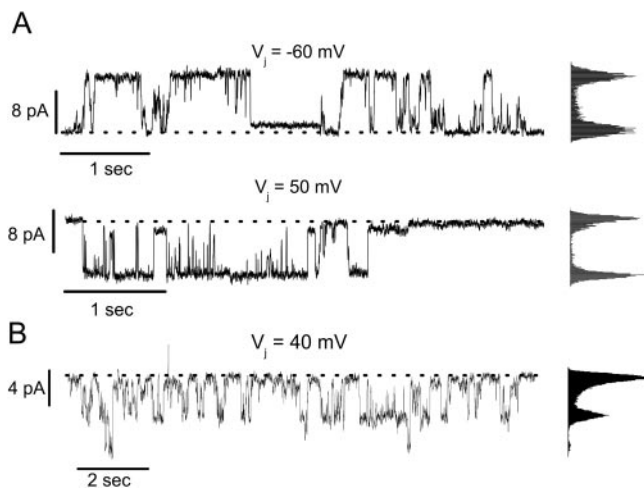


FIGURE 5. Single-channel currents in epithelial cells from wild-type and Cx46 knockin mice. (A) Recordings of unitary currents from two different wild-type epithelial cell pairs at transjunctional voltage (V_j) gradients of -60 mV and $+50$ mV. Single-channel currents were large in amplitude and exhibited transitions between open, closed, and subconductance states. Amplitude histograms of the recordings, illustrated on the right of each trace, indicate an open state conductance of approximately 200 pS. The dotted lines indicate the fully closed state. (B) Recording of unitary currents from an epithelial cell pair isolated from Cx46 knockin lenses at a V_j of 40 mV. Amplitude histograms of the recordings, illustrated on the right of each trace, indicate an open state conductance of approximately 128 pS. The dotted lines indicate the fully closed state.

the closed state, respectively, and yielding a unitary conductance of 193 pS. Similarly, at a transjunctional voltage of 50 mV, the unitary current amplitude was approximately 10.2 pA, equivalent to unitary conductance of 204 pS. The single-channel current-voltage relationships constructed from four different cell pairs yielded a slope conductance of 202 pS, a value similar to that measured in N2A cells expressing Cx50.¹⁹

In epithelial cells of Cx46 knockin mice, the amplitude of single-channel currents was smaller than that observed in wild-type epithelial cells. Figure 5B illustrates a long recording from a weakly coupled cell pair obtained from Cx46 knockin lenses. The amplitude histogram for this recording, illustrated on the right, contains distinct peaks at 5.1 pA and 0 pA, corresponding to the open and closed state, respectively, and yielding a unitary conductance of 128 pS at a V_j of $+40$ mV. This value is 8% to 10% lower than that of Cx46 homotypic junctions in exogenous expression systems but higher than the unitary conductance for Cx43 gap junctions in N2A or HeLa cells. Cx43 and Cx46 can form heterotypic gap junctions,³¹ and the unitary conductance of heterotypic channels is generally intermediate between the values of the homotypic forms.^{29,32} Hence, the recording depicted in Figure 5B may represent a Cx43–Cx46 heterotypic junction. Taken together, these results provide further biophysical evidence that Cx50 contributes to junctional conductance in epithelial cells in wild-type lenses from P3 mice and that Cx46 functionally replaces Cx50 in knockin epithelial cells.

Contribution of Cx50 to Gap Junction Coupling in Epithelial Cells Is Reduced with Age

The number and location of mitotically active lens cells is highly dependent on the age of the mouse.^{3,33} From P2 to P3, such cells are found across the entire lens epithelium, whereas after P7, most of the mitotically active cells are found only in the germinative zone near the lens equator. Because previous

studies indicate that the growth defect in Cx50 knockout mice is manifested during the first postnatal week, one might expect that the contribution of Cx50 to epithelial cell coupling is highest during the first postnatal week and decreases in older lenses. To test this prediction, we determined the effect of quinine on junctional currents in cell pairs obtained from P28 and P12 lenses.

The sensitivity of junctional currents in P28 epithelial cells to quinine was markedly reduced compared with cells from P3 (Fig. 6A). The magnitude of reduction caused by quinine ranged from 20% to 30% in P28 epithelial cells (Fig. 6B), compared with 60% to 70% reduction in P3 epithelial cells (see Fig. 4). Although the contribution of Cx50 was reduced in the adult, this was not because of a reduction in Cx50 protein levels. Western blot analyses indicated that Cx50 protein expression in capsules obtained from different developmental ages was similar (data not shown). In addition, some P28 cell pairs exhibited less than a 15% reduction in junctional currents on application of quinine, in contrast to P3 epithelial cells in which more than 95% of the cells were responsive to the drug. As expected, junctional conductance in P28 epithelial cells from knockin lenses was not affected by quinine application (Fig. 6B).

Measurement of single-channel properties indicated that Cx43 dominantly contributed to coupling in P28 cells. The

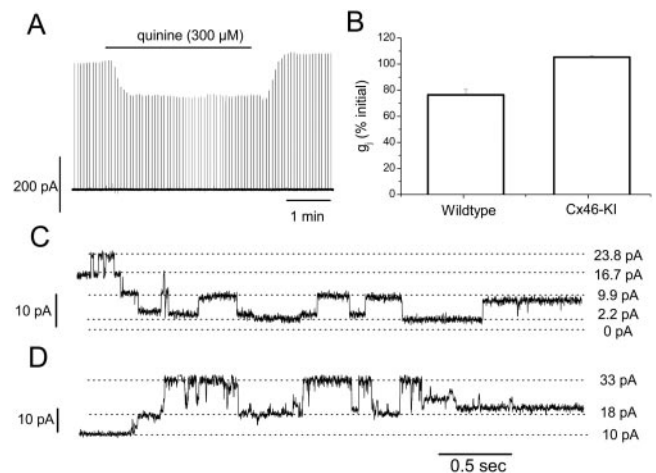


FIGURE 6. Characteristics of coupling in epithelial cells isolated from P28 lenses. (A) Effect of quinine on junctional currents from a P28 epithelial cell pair isolated from wild-type lenses. As in cells isolated from P3 lenses, quinine caused a reversible reduction in the junctional current. However, the magnitude of the quinine-induced reduction at P28 was approximately 24% , a value lower than that observed in cells isolated from P3 lenses, suggesting that older cells are most likely coupled by Cx43 gap junctions. (B) Bar graph summarizing the effect of quinine on coupling in wild-type and Cx46 knockin epithelial cells from P28 lenses. Junctional conductance (g_j) in P28 wild-type cells was reduced by 24.5% in the presence of quinine. Junctional conductance in epithelial cells from P28 knockin lenses was not affected by quinine application. Each bar represents the mean \pm SEM of three to nine cell pairs. (C) Single-channel currents in P28 epithelial cells resemble those formed by Cx43 gap junctions. Recordings of single-channel currents from an epithelial cell pair at a V_j of -70 mV. Application of a V_j gradient of -70 mV caused each of the three channels in this cell pair to progressively close to a nonzero steady state level (top trace). Amplitudes of the currents at various levels are indicated on the right of each trace. Transitions of the three open channels corresponded to a unitary conductance of 100 to 110 pS, a value similar to the unitary conductance of Cx43 gap junction channels. (D) In the same cell pair, openings and closings of a large conductance channel (approximately 200 pS) was observed. The large amplitude of the unitary current was similar to that observed for Cx50 gap junctions in exogenously transfected cells.

properties of macroscopic and unitary currents in older epithelial cells were similar to those observed in Cx43-transfected cells. Unitary currents in a weakly coupled cell pair isolated from P28 lenses are shown in Figure 6C. Application of a transjunctional voltage gradient of -70 mV caused each of the three channels in this cell pair to progressively close to a nonzero steady state level (Fig. 6C). The amplitude of transitions of the three open channels were approximately 7.1 pA, 6.8 pA, and 7.7 pA, corresponding to a unitary conductance of 100 to 110 pS, a value similar to the unitary conductance of Cx43 gap junction channels. Similar results were obtained in four different cell pairs. In addition, in some cell pairs, opening of a large conductance channel (approximately 200 pS), most likely corresponding to Cx50 gap junctions, was occasionally observed (Fig. 6D). Although such large conductance channels were only rarely observed, the results indicated that Cx50 is not entirely absent in these cells, consistent with the pharmacologic studies illustrated in Figure 6A. These results indicated that the magnitude and the proportion of cells expressing functional Cx50 gap junctions were strongly reduced in the older lens epithelial cells.

In lenses from P12 mice, the contribution of Cx50 to total coupling was also reduced compared with that of P3 lenses. The magnitude of reduction caused by quinine ($44.6\% \pm 10.2\%$; $n = 7$) was not as low as found for older epithelial cells but it was still significantly lower than the 60% to 70% reduction found in P3 epithelial cells (Fig. 7). As with P3 epithelial cells, the pharmacologic studies were validated by analysis of single-channel currents, which showed channel sizes corresponding to Cx50 gap junctions persisted in these cells (Fig. 7B). In this particular example, recordings of single-channel currents in response to voltage ramps from -100 mV to $+100$ mV indicated a unitary conductance of 223 pS, indicating that the contribution of Cx50 is highest in P3 epithelial cells but progressively declines with age thereafter.

DISCUSSION

Using a combination of immunocytochemical, pharmacologic, and electrophysiological methods, we demonstrated that Cx50 is strongly expressed in the lens epithelium and contributes to epithelial coupling in a developmentally regulated manner. In addition, our results indicate that functional activity contributed by Cx50 is required for proliferation in the central epithelium during postnatal lens growth. Coupling between epithelial cells isolated from P3 lenses, a developmental time point involving a large number of proliferating cells in both the central and the equatorial epithelium, was primarily provided by Cx50, as evidenced by the strong reduction (more than 60%) in junctional conductance elicited by quinine, a drug that blocks Cx50 but not Cx43 or Cx46. In contrast, the contribution of Cx50 to total coupling in older epithelial cells (P12 or P28) in which BrdU labeling is reduced and restricted to the equatorial zone was markedly lower than in early postnatal cells. An additional result of this study was that Cx46 knockin epithelial lacked proliferating cells in the central epithelium on P3, even though the absolute magnitude of coupling was similar to that of wild-type cells, indicating that Cx46 cannot substitute for Cx50 in facilitation of lens cell division, especially in the central epithelium. These results establish that Cx50 influences epithelial cell proliferation as an active participant in junctional communication before it is replaced by Cx43 as the principal epithelial cell connexin in an age-dependent manner.

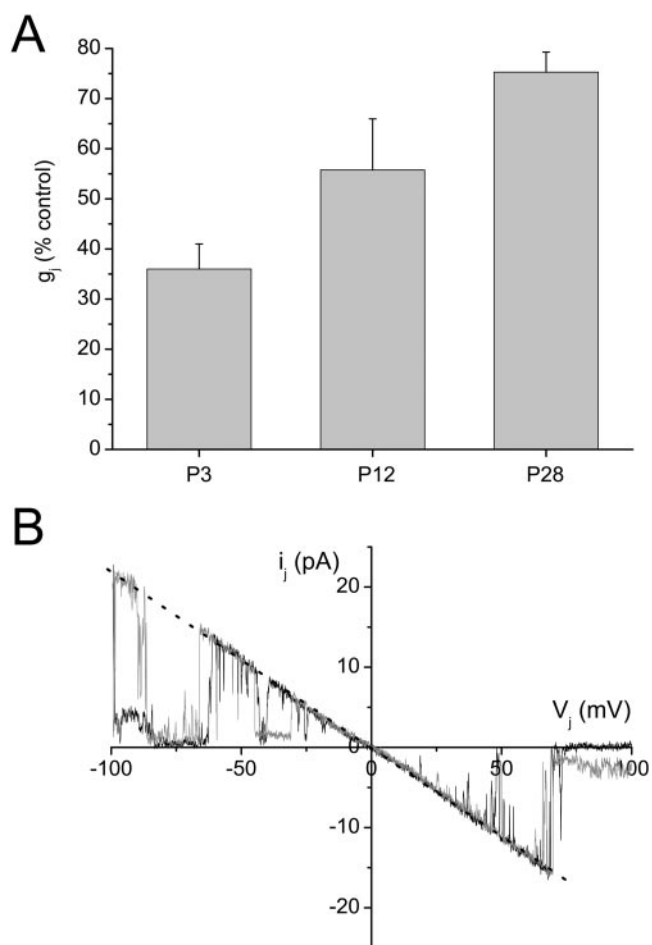


FIGURE 7. Characteristics of coupling in epithelial cells isolated from P12 lenses. **(A)** Bar graph summarizing the effect of quinine on coupling in epithelial cells from P12 lenses (*middle bar*). Junctional conductance (g_j) was reduced by $44\% \pm 10.2\%$ in the presence of quinine ($n = 7$). The effect of quinine in P3 and P28 epithelial cells is replotted from Figures 4 and 6 to illustrate the age-dependent decline in the contribution of Cx50 to epithelial junctional coupling. **(B)** Recordings of unitary currents in epithelial cell pairs from P12 lenses constructed from the responses of junctional membrane to a series of ramps from -100 to $+100$ mV. The unitary conductance of the main state ($\gamma_{j,\text{main}}$) measured as the slope of the current-voltage relationship (*dotted line*) was 223 pS, a value similar to the unitary conductance of Cx50 gap junction channels.

Cx50 Channel Properties Are Age Dependent and Correlate with Cell Proliferation

Lens growth results from the programmed proliferation of epithelial cells before their differentiation into new fiber cells. In the mouse epithelium, postnatal proliferation peaks between P2 and P3. At this time, cells throughout the epithelium undergo proliferation, with doubling times of approximately 1 day.^{3,54} However, after the first week, both the number and the rate of proliferating cells decrease and become spatially restricted to the equatorial region of the lens.³³ Our results indicate that the differences in proliferation patterns between postnatal and adult lens epithelial cells correlate with a progressive shift in the connexin isoform, primarily contributing to gap junctional communication from Cx50 to Cx43. During the first week of growth, Cx50 contributes more than 60% of total epithelial cell coupling, whereas by 4 weeks of age, the contribution of this connexin declines to 25% on average. These data suggest that the optimal proliferation of epithelial

cells on P2 and P3 requires the unique functional properties of Cx50, whereas epithelial cell function in older lenses is not as dependent on Cx50.

How Does Cx50, but Not Cx46, Positively Influence Proliferation?

We have documented a role for functional coupling provided by Cx50 in stimulating proliferation in the central epithelium of the early postnatal lens. Expression of Cx46 in place of Cx50 by genetic knockin could not restore central epithelial proliferation, suggesting that more than simple ionic coupling, which would have been provided by either connexin, was required for the propagation of mitotic stimulation.³ Replacement of Cx50 with Cx46 resulted in significantly fewer dividing cells in the central and the equatorial epithelium during the days of maximal postnatal lens cell proliferation (Fig. 1). Stimulated lens mitosis has been linked to growth factors, which in turn activate intracellular signaling cascades.³⁵⁻³⁸ We speculate that Cx50, but not Cx46, selectively mediates intercellular propagation of the second messengers of growth factor signal transduction, resulting in greater recruitment of neighboring cells into mitosis, particularly in the central epithelium. Differences between connexin isoforms in the permeability of second messengers and growth signals have been documented.³⁹ Alternatively, growth factors may have an effect on epithelial cell coupling by directly gating Cx50 but not Cx46 channels. For example, low concentrations of FGF were shown to induce an increase in coupling in chick lens cultures³⁷ through sustained activation of the ERK pathway. Additional studies will be required to distinguish between these possibilities.

It is intriguing to speculate whether increasing Cx50 in epithelial cells would augment epithelial cell proliferation. One transgenic model of Cx50 overexpression resulted in smaller lenses with cataract,⁴⁰ though the promoter used in this study targeted lens fiber expression and would not be expected to be active in the epithelial cells, complicating any interpretation of these data with regard to the present study. We have previously shown that reducing Cx50 expression in heterozygous knockout or knockin (Cx50^{+/-} or Cx50^{+/46}) mice did not result in reduced lens size,^{8,41} suggesting that one allele of Cx50 is sufficient to promote normal growth. Future experiments testing the effect of overexpression of Cx50 in epithelial cells could be attempted by knocking the Cx50 coding sequence into the Cx43 gene locus.

Discrepancy with Previous Results

In contrast to our results, previous electrophysiological studies indicated that the lens epithelium expressed only Cx43. For example, Donaldson et al.^{22,23} found that properties of gap junction channels in a sheep epithelial cell line matched those formed by Cx43 in expression systems and concluded that epithelial cells express only Cx43. Similarly, recordings of unitary junctional currents in amphibian epithelial cells indicated that Cx43 was the predominant connexin in the epithelium.⁴² However, most of these recordings were obtained from immortalized lens epithelial cell lines or adult lenses, whereas our data showed that the expression of Cx43 predominates over Cx50. The main difference between these studies and ours is that we used lens epithelial cells acutely isolated from mice at different developmental stages instead of lens epithelial cell lines. A second difference is the availability of a Cx50-specific blocker such as quinine, which allowed us to determine the relative contributions of Cx43 and Cx50. The presence of Cx50 in the lens epithelium was also documented by immunofluorescence studies of isolated lens capsules (Fig. 2) and was consistent with previous studies. For example, Dahm et al.²⁷ demonstrated that Cx50 was coexpressed with Cx43 in

lens epithelial cells. In addition, analysis of LacZ expression in Cx50 knockout mice with a knockin LacZ reporter gene indicated extensive labeling in epithelial cells in newborn and adult mice.⁹ Taken together, the results indicate that Cx50 contributes robustly to gap junctional coupling in lens epithelial cells, particularly during the first postnatal week.

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