

Stimulation of Lens Cell Differentiation by Gap Junction Protein Connexin 45.6

Sumin Gu, X. Sean Yu, Xinye Yin, and Jean X. Jiang

PURPOSE. The present study was undertaken to explore the roles gap junctions play in lens epithelial cell differentiation.

METHODS. Recombinant retroviruses expressing three chick lens connexins (Cx)—Cx43, Cx45.6, and Cx56—were prepared and used to infect isolated chick lens primary cultures. The expression and distribution of proteins was determined using immunoblots and confocal immunofluorescence microscopy. Intercellular couplings were assessed by single cell microinjection and scrape-loading dye transfer, and cell proliferation was evaluated by [³H]thymidine labeling.

RESULTS. Of the three lens connexins, only the cultures overexpressing exogenous Cx45.6 displayed the advancement of lens epithelial-fiber cell differentiation. The lentoids, a unique morphologic structure that is an indicative of lens fiber formation, were formed earlier in Cx45.6 overexpressed cultures; however, the rate of lens cell proliferation was not affected. The expression of the lens differentiation marker proteins, major intrinsic protein (MIP) and δ -crystallin, was also increased in Cx45.6-overexpressing cells. The cells overexpressing Cx45.6 displayed similar levels of intercellular couplings as did the controls. Moreover, exogenously expressed connexins were mostly colocalized with their endogenous counterparts and the overexpression of Cx45.6 had no impact on the expression of endogenous Cx43 and Cx56.

CONCLUSIONS. These results suggest that Cx45.6 plays an important role in stimulating lens cell differentiation and fiber formation, which is different from the other lens connexins, Cx43 and Cx56. This stimulatory effect is independent of gap junction-mediated intercellular communication and lens cell proliferation. (*Invest Ophthalmol Vis Sci.* 2003;44:2103-2111) DOI:10.1167/iovs.02-1045

The eye lens, suspended from the ciliary body, retains a stem cell population that continues to proliferate and differentiate throughout the life of the organism.¹ Epithelial cells covering the anterior surface of the lens fold posteriorly at the equator, where they are continuous during both differentiation and formation of the lens fibers. The fibers in the center of the lens (constituting >90% of the total lens volume) are coupled with cells at the lens surface through a highly developed gap-junction-mediated intercellular communication network. This extensive network facilitates the exchange of ions and

metabolites throughout the lens, maintaining osmotic and metabolic homeostasis and lens transparency.^{2,3}

Gap junctions are clusters of transmembrane channels that connect the cytoplasm of adjacent cells and allow small molecules ($M_r \leq 1000$ Da), such as metabolites, ions, and second messengers, to translocate from cell to cell.⁴ The structural components of gap junctions are members of a family of membrane proteins known as connexins, which consist of four conserved transmembrane domains and two conserved extracellular loops, whereas sequences in the cytoplasmic loop, especially those in the cytoplasmic tail, are unique. Three connexins have been identified in the mammalian lens—Cx43, Cx46, and Cx50⁵—of which Cx46 and Cx50 are mainly colocalized in the lens fibers. Recently, several mutations of Cx46 and Cx50 have been identified that are directly linked to human autosomal dominant congenital cataracts.⁶ Moreover, studies have shown that lens cataracts develop in mice without either of the lens fiber connexins Cx50 or Cx46^{7,8}; however, only mice deficient in Cx50 exhibit a dramatic decrease in lens size⁸ and delayed lens fiber maturation.⁹ The developmental defect caused by the absence of Cx50 cannot be corrected with targeted replacement by Cx46 knock-in mice.¹⁰ These results suggest that two connexins coexpressed in lens fibers may play different roles in lens formation and function and that Cx50 appears to be involved in lens development.

The primary chick lens cultures have been used extensively as an ideal *in vitro* model that closely mimics the differentiation process of lens cells *in vivo*. The monolayer lens epithelial cells gradually differentiate into structures called lentoids with features of differentiated lens fibers.¹¹ However, in other species, such as in rodent or cattle, lens primary cultures only partially differentiate as exhibited by incomplete phosphorylation of connexins, compared with *in vivo* phosphorylation.¹² Three connexins have been identified in the chick lens: Cx43,¹³ Cx45.6,¹⁴ and Cx56,¹⁵ the orthologues of rodent Cx43, Cx50, and Cx46, respectively. Cx45.6 and Cx56, which are expressed predominantly in lens fiber cells, are also found to a lesser extent in junctions between epithelial cells in lens organs¹⁶ and in lens primary cultures.¹⁷ Cx45.6 and Cx56 are colocalized and form heteromeric hemichannels that are also called heteromeric connexons.¹⁸

In several studies, results have implied the involvement of gap junctions in various developmental processes.¹⁹ Findings in studies using anti-connexin antibodies, pharmacological inhibitors, dominant negative connexin mutants, and antisense constructs indicate a role for gap junctions in early vertebrate embryogenesis²⁰ and limb patterning.^{21,22} Conversely, establishment of gap-junction-mediated intercellular communications after transfection of connexin genes into cells is reported to increase the expression of tissue-specific genes in certain cell lines.²³ The role of gap junction communication in lens development is supported by the observation that transformation of lens primary cell cultures with nonspecific Rous sarcoma virus inhibits intercellular coupling and lens cell differentiation.²⁴ In contrast to this observation, Le and Musil¹⁷ found that the inhibition of intercellular communication by a gap junction blocker does not affect epithelial-fiber cell differentiation and formation of lentoids in lens primary cultures,

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suggesting that functional gap junction channels are not essential for lens cell differentiation. However, knocking out the lens fiber connexin gene *Cx50* in mice results in decreased lens size.⁸ Therefore, the exact molecular mechanism of the involvement of lens connexins in epithelial-fiber cell differentiation and lens development remains largely unknown.

In this study, primary cultures of chick lens, which closely resemble *in vivo* epithelial-fiber cell differentiation, were used as an experimental model to explore mechanistic aspects of the roles of connexins in lens cell differentiation. Because of the difficulties of traditional gene transfection approaches in expressing proteins in primary cultures, expression of exogenous connexins was achieved by a retroviral approach that we have successfully used in chick lens.²⁵ Three lens connexins were overexpressed efficiently in these primary cultures by retroviral infection. We show that only one of the lens connexins, *Cx45.6*, stimulated epithelial-fiber cell differentiation, and major intrinsic protein (MIP) and δ -crystallin expression; the other two types of lens connexins do not. Moreover, expression of exogenous *Cx45.6* did not affect lens cell proliferation, intercellular coupling, or the expression of endogenous *Cx43* and *Cx56*.

MATERIALS AND METHODS

Reagents

Fertilized chicken eggs were obtained from SPAFAS (Roanoke, IL) or Tyson Hatchery (Gonzalez, TX), and incubated for 11 days in a humidified 37°C incubator. Anti-*Cx43*, anti-*Cx45.6*, and anti-*Cx56* antibodies were raised and affinity purified, as previously reported.²⁶ P27 (anti-GAG) antibody was from SPAFAS. Anti-chick MIP monoclonal antibody²⁷ was a generous gift from Erica Tenbroek and Ross Johnson at the University of Minnesota (St. Paul, MN). Anti-chick δ -crystallin antibody was generously provided by Joram Piatigorsky at the National Eye Institute (Bethesda, MD). Rhodamine-conjugated goat anti-mouse IgG was purchased from Pierce Chemical Corp. (Rockford, IL); fluorescein-conjugated goat anti-rabbit IgG from ICN (Costa Mesa, CA); paraformaldehyde (16% stock solution) from Electron Microscopy Science (Fort Washington, PA); trypsin and tissue culture reagents from Invitrogen (Carlsbad, CA); nitrocellulose membrane from Schleicher & Schuell (Keene, NH); fetal bovine serum (FBS) from Hyclone Laboratories (Logan, UT); bicinchoninic acid (BCA) microprotein assay kit from Pierce Chemical Corp.; a chemiluminescence kit (ECL) from Amersham Pharmacia Biotech (Piscataway, NJ); and biocytin was from Molecular Probes (Eugene, OR). All other chemicals were obtained from either Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Preparation of Recombinant Retroviral Constructs Containing Lens Connexins and Generation of High-Titer Retroviruses

The procedure for cloning the full-length lens connexins *Cx43*, *Cx45.6*, and *Cx56* into the retroviral vector-RCAS(A) was based on a protocol previously described.²⁸ Briefly, the connexin was first cloned to an adapter plasmid to facilitate the proper restriction sites and then to the RCAS(A) retroviral vector driven by viral promoter. DNA fragments containing connexins with their carboxyl termini in-frame with the FLAG epitope tag sequence (DYKDDDDK) were prepared by polymerase chain reaction. The primers for PCR were synthesized at the University of Texas Health Science Center at San Antonio (UTHSCSA) DNA Core Facility. To ensure the sequence of these constructs were correct, the DNA was also sequenced at the UTHSCSA DNA Core Facility. The procedure for preparation of high-titer retroviruses was based exactly on a previously published report.²⁵

Primary Lens Cell Cultures

Lens cell cultures were prepared by a modified method, as described previously.²⁹ Lenses were excised from day-11 chicken embryos and

washed with TD buffer (140 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 5 mM glucose, and 25 mM Tris [pH 7.4]). The ciliary epithelium and vitreous body attached to the lens were removed by digesting the lenses with 0.1% trypsin in TD buffer at 37°C for 30 minutes. The digested lenses were washed with TD buffer, and cells were separated by pipetting up and down through a 5-mL pipette in medium 199 containing 10% fetal bovine serum. Individual lens cells were collected by centrifugation at 150g for 5 minutes and resuspended in medium 199. The cells were counted and plated at 1×10^6 cells per 35-mm cell culture dish. The cultures were incubated at 37°C, 5% CO₂ and fed every other day. In the beginning of culturing, only monolayer lens epithelial cells proliferate on the culture plates, but not fiber cells. The initiation and formation of lentoid bodies occurred around 4 days in primary lens cultures.

Retroviral Expression of Exogenous Connexins in Lens Primary Cell Cultures

Primary cell cultures derived from embryonic day-11 chick lenses were prepared as described earlier. On the second day after cell seeding and thereafter, the cultures were infected with high-titer retroviruses ($1-5 \times 10^8$ colony-forming units [cfu]/mL), RCAS(A), RCAS(A)-*Cx43*, RCAS(A)-*Cx45.6*, or RCAS(A)-*Cx56*. Twenty-four hours after infection, the cultures were washed and fed with fresh culture medium. The cultured cells were fed with fresh medium every other day and were then used for further immunofluorescence studies, Western blot studies, or dye-transfer assays.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Crude membranes from embryonic chick lenses and lens primary cultures were prepared as described.¹³ An equal amount of samples determined by a micro-BCA assay was analyzed on 10% SDS-PAGE, according to the method of Laemmli.³⁰ Western blot analyses of proteins were performed by probing with anti-FLAG (1:1000), affinity-purified anti-lens connexin (1:500 dilution), anti- δ -crystallin (1:100 dilution), and anti- β -actin (1:5000 dilution) antibodies. The anti-connexin antibodies detected both endogenous and exogenously expressed lens connexins, whereas anti-FLAG antibody detected only exogenous connexins introduced by retroviral infection. The primary antibodies were detected, either by using alkaline phosphatase-conjugated secondary antibodies followed by a color reaction or by using peroxidase-conjugated secondary antibodies followed by chemiluminescence detection, according to the manufacturer's instruction (ECL; Amersham Pharmacia Biotech). For chemiluminescence detection, the membranes were exposed to autoradiograph films (X-OMAT; Eastman Kodak, Rochester, NY) and detected by fluorography.

Immunofluorescence and Confocal Laser Microscopy

Polyclonal anti-lens connexin antibodies and monoclonal anti-FLAG antibody M2 were used as primary antibodies to detect endogenous and exogenous connexins, respectively. Because MIP and δ -crystallin are lens fiber marker proteins, anti-MIP and anti- δ -crystallin antibodies were used to distinguish truly differentiated lentoids from multilayered lens cells.¹⁷ Embryonic lens cell primary cultures planted on glass cover slides were washed with PBS, fixed in 2% paraformaldehyde in PBS for 30 minutes, washed again with PBS, and then blocked with blocking solution (2% normal goat serum, 2% fish skin gelatin, 0.25% Triton X-100, and 1% bovine serum in PBS) for 30 minutes at room temperature. The cultures were then incubated with the primary antibodies (1:500 dilution for anti-connexins, 1:100 for anti-MIP and anti- δ -crystallin, and 1:1000 dilution for anti-FLAG as recommended by the manufacturer in blocking solution) for 12 hours at 4°C. These cultures were washed three times with PBS and then incubated with rhodamine- or fluorescein-conjugated secondary antibodies, anti-rabbit for lens connexins (1:500 dilution) and anti-mouse for the FLAG

epitope, MIP, and δ -crystallin (1:500 dilution), for 2 hours at room temperature. The cultures were washed three times with PBS and preserved in mounting medium (Vector Laboratories). The specimens were analyzed using a confocal laser scanning microscope (Fluoview; Olympus, Tokyo, Japan). FITC fluorescence was excited at 488-nm by an argon laser and rhodamine was excited at 543-nm with a HeNe-G laser. The emission filters used were BA505-525 for FITC fluorescence and BA610 for rhodamine (Olympus).

The primary cultures of chick embryonic lens cells were plated and maintained for up to 2 weeks. The morphology of cultured cells and lentoid structures were documented daily using an Olympus phase-contrast microscope (Tokyo, Japan) and the number of lentoid bodies and the size of FITC-labeled, MIP-positive areas were counted and quantified. Five independent experiments with five separate retroviral infections were performed. For quantification of MIP-positive stained areas in lens cultures, 10 fluorescence images were taken randomly at various regions in each individual culture dish and the average size of MIP staining area versus the whole image area, a measurement of MIP expression levels, were quantified by using the UTHSCSA ImageTool Image Analysis software (<http://ddsdx.uthscsa.edu/dig/itdesc.html>; provided in the public domain by UTHSCSA) to determine the degree of the differentiation of cell cultures with different retroviral treatments.

Cell Proliferation Assay by [³H]thymidine Labeling

Cell proliferation assay was modified from a previously published procedure.³¹ When the primary lens cultures infected by retroviruses, RCAS(A) or RCAS(A)-Cx45.6, reached 60% to 70% confluence, cells in each culture plate (35 mm) were labeled with 6 μ Ci [³H]thymidine in 1.5 mL of culture medium for 24 hours. After labeling, cells were washed three times with cold PBS and incubated in 10% trichloroacetic acid (TCA) solution on ice for 15 minutes. After the removal of TCA solution, cells were incubated with 0.6 mL of 0.1 N NaOH on ice for 10 minutes, after which the DNA extract in the NaOH solution was collected. Six hundred microliters of 0.1 N HCl was used to rinse the dish and was then mixed with the NaOH solution containing the DNA extract. Twenty microliters of the labeling mixture was mixed with 10 mL of scintillation fluid for radioactivity measurement.

Dye-Transfer Assay for Gap Junction Intercellular Communication

A cell microinjection assay was performed as described previously.³² Briefly, Lucifer yellow (LY; M_r : 457 Da; 0.2%), which can penetrate through gap junction channels, was microinjected into cells of a 5-day lens primary cell culture. The transfer of the dye was visualized using a phase-contrast fluorescence microscope (Axioscope; Carl Zeiss, Jena, Germany), in which LY could be detected by using the filter set for fluorescein. Five minutes after the donor cell was injected, the distance between the original dye-injected cell and the most distal cell receiving the dye was measured. The degree of dye-transfer was determined based on the data collected from three independent experiments involving three separate infections with corresponding retroviruses. In each experiment, five cells were injected with dyes.

The scrape-loading dye-transfer assay was modified based on a published procedure.^{17,33} In this method, cells were scratched in the presence of two types of fluorescence dyes: rhodamine dextran (RD; M_r : 10 kDa) and either LY or biocytin (M_r : 372 Da). RD is too large to pass through gap junction channels and therefore served as a tracer dye for the cells originally receiving the dye. Five-day lens epithelial cell cultures were washed three times with Hanks' balanced salt solution (HBSS) plus 1% BSA for 5 minutes each. Then, 1% RD and either 1% LY or 1% biocytin dissolved in PBS were applied to the cells that were subsequently scraped lightly with a 26-gauge needle. After incubation for 10 minutes, cells were washed with HBSS three times, then twice with PBS, and finally fixed in fresh 2% paraformaldehyde (from 16% stock) for 20 minutes. For biocytin visualization, fixed cells

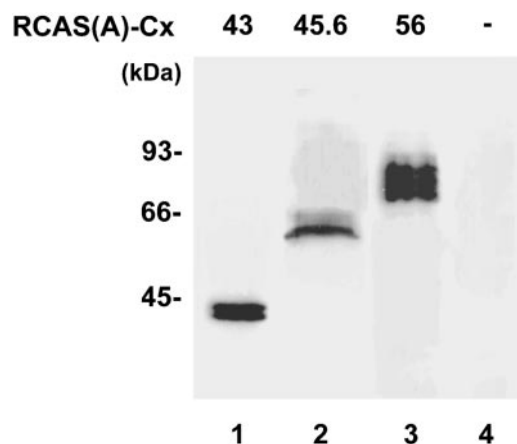


FIGURE 1. Immunoblot of RCAS(A)-connexin constructs expressed in primary cell cultures of embryonic chick lens. Primary lens cultures from day-11 chick embryos were infected with the high-titer retroviruses RCAS(A)-Cx43 (lane 1), RCAS(A)-Cx45.6 (lane 2), RCAS(A)-Cx56 (lane 3), and RCAS(A) (lane 4). After 7 days of infection, embryonic cultured cells were lysed, and crude membranes were isolated. These membrane preparations were analyzed on SDS-PAGE, and nitrocellulose replicas were probed with anti-FLAG antibody and visualized with alkaline phosphatase colorimetric reaction.

were permeabilized for 15 minutes with PBS containing 0.1% Triton X-100, 0.2% BSA, and 5% normal goat serum followed by incubation with avidin-FITC (1:400) for 2 hours at room temperature. The dye-transfer results were examined with a fluorescence microscope in which LY and avidin-FITC could be detected with the filter set for fluorescein, and RD could be detected with the filter set for rhodamine. The degree of dye transfer was observed based on three independent experiments with three separate infections with corresponding recombinant retroviruses.

Statistical Analysis

Data were analyzed with one-way ANOVA and Student-Newman-Keuls multiple comparison tests with a biostatistics program (InStat; Graph-Pad Software, Inc., San Diego, CA). In Figures, data are presented as the mean \pm SD of at least three determinations. Asterisks in the figures indicate the degree of significant differences compared with the control (* P < 0.05; ** P < 0.01; *** P < 0.001).

RESULTS

Retroviral Expression and Colocalization of Exogenous Connexins

The exogenous expression of each lens connexin in lens primary cultures was attempted by using our previously developed retroviral approach.²⁵ High-titer retroviruses—RCAS(A), RCAS(A)-Cx43, RCAS(A)-Cx45.6, and RCAS(A)-Cx56—were used to infect monolayer primary lens cultures. Seven days after the infection, the expression of the three exogenous connexins was clearly demonstrated by Western blot analysis (Fig. 1) and immunofluorescence studies (Fig. 2). Exogenous Cx43 (Fig. 1, lane 1), Cx45.6 (Fig. 1, lane 2), and Cx56 (Fig. 1, lane 3) were abundantly expressed in lens primary cultures, as detected by anti-epitope tag FLAG antibody. No band was detected from cultures infected with retrovirus, RCAS(A) (Fig. 1, lane 4). Exogenous connexins were expressed in multiple migrating forms, as detected by SDS-PAGE. Similar migrating patterns have been shown for their endogenous counterparts, expressed in primary lens cultures, as a result of posttranslational phosphorylation.^{34,35} Immunofluorescence experiments using anti-FLAG epitope tag antibody revealed the localization

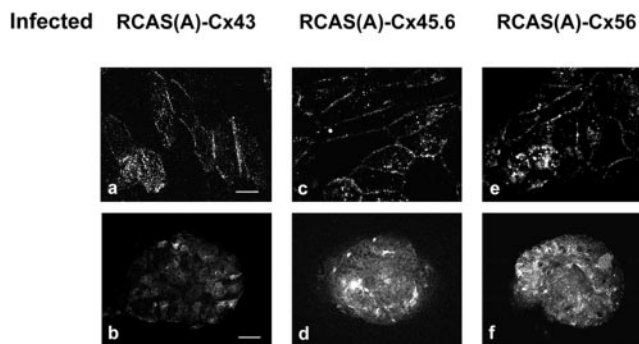


FIGURE 2. Immunofluorescence staining of lens primary cultures expressing exogenous connexins. Seven days after infection, epithelial cells (a, c, e) and lentoid structures (b, d, f) of primary cultures expressing exogenous Cx43 (a, b), Cx45.6 (c, d), and Cx56 (e, f) were fixed and labeled with anti-FLAG antibody. The primary antibody was detected by fluorescein-conjugated anti-mouse IgG. The immunostaining was visualized by confocal fluorescence microscopy. Scale bar: (a, c, e) 10 μm ; (b, d, f) 80 μm .

of exogenously expressed lens connexins (Fig. 2). All three exogenous connexins were expressed in lens epithelial cells, which were revealed by immunofluorescence signals as punctuate spots surrounding the cells (Figs. 2a, 2c, 2e), which is the typical expression pattern of gap junctions. Exogenous Cx45.6 and Cx56 were also expressed in abundance within lentoid bodies (Figs. 2d, 2f). Cx43, in comparison with Cx45.6 and Cx56, was expressed to a lesser extent in the lentoid bodies, and the expression was mostly localized close to the edges and surface areas of the lentoids (Fig. 2b). After 3 days of infection, more than 95% of the cells on the culture plate contained exogenously expressed connexins.

Immunofluorescence experiments with anti-epitope tag (FLAG) and anti-connexin antibodies revealed that exogenously expressed lens connexins were mostly colocalized with endogenous connexins (Fig. 3). Both Cx45.6 and Cx56

were abundantly expressed within lentoid bodies (Figs. 3a, 3e). Exogenous Cx45.6 and Cx56 expressed by retroviral infection were mostly localized at the same junctional plaques as their endogenous counterparts in lentoid bodies, although a much lower level of nonoverlapping localization was also observed (Figs. 3b–d, 3f–h). Colocalization of exogenous Cx43 with its endogenous counterpart was also observed (data not shown).

Together, these results suggest that the retroviral expression approach efficiently expresses exogenous lens connexins in lens primary cultures and exogenous connexins were mostly colocalized with their endogenous counterparts.

Effects of Cx45.6, Cx43, and Cx56 on Lens Cell Differentiation

The lens cell differentiation processes associated with lentoid formation in lens primary cultures were compared and documented in the presence and absence of the expression of exogenous lens connexins. The second day after cell plating, high-titer retroviruses, RCAS(A) (vehicle), -Cx43, -Cx45.6, and -Cx56 were used to infect lens primary cultures. The total number of lentoids formed in the vehicle-treated culture or culture expressing exogenous connexins was counted and quantified at various culture periods (Fig. 4a). The initiation and induction of lentoid bodies occurred at approximately 4 days in primary lens cultures. At the various infection times tested, the total number of lentoids increased close to onefold when overexpressing Cx45.6 compared with Cx43- and Cx56-overexpressing or RCAS(A) vehicle-treated cultures (Fig. 4a). [^3H]thymidine labeling assay showed that, compared with the control, there was no significant change in the rate of cell proliferation for cultures overexpressing Cx45.6 (Fig. 4b), indicating that stimulation of lentoid formation by Cx45.6 was not related to the changes in cell proliferation.

Effect of Cx45.6 Overexpression

To determine the extent of lens cell differentiation, the expression of the lens differentiation markers, MIP and δ -crystallin,

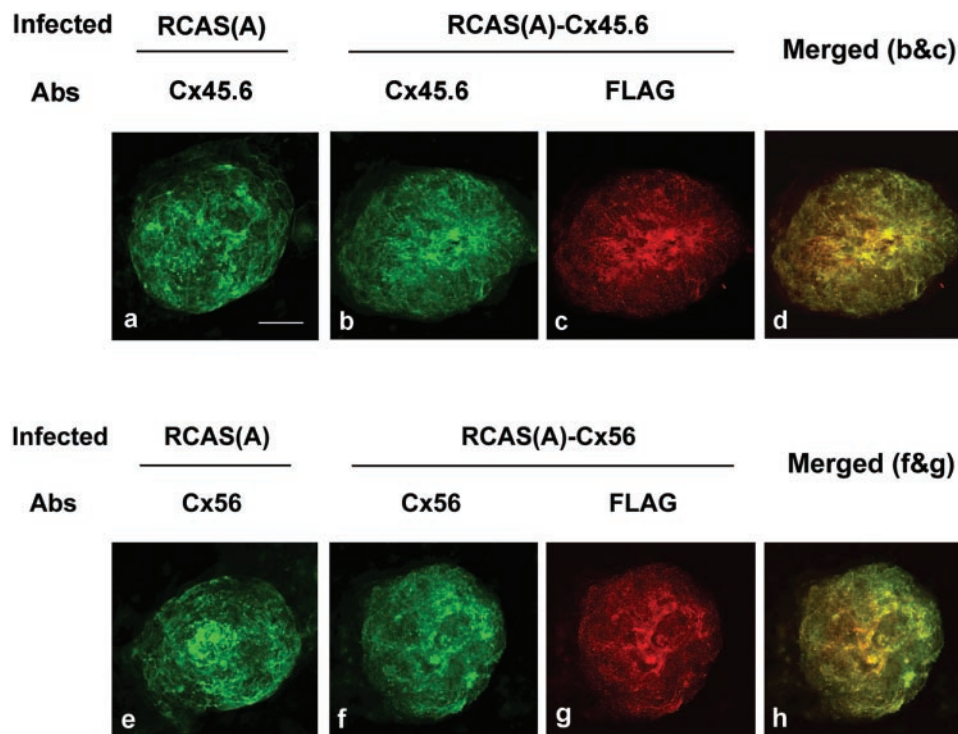


FIGURE 3. Exogenous connexins expressed by retroviral infection were colocalized with endogenous connexins expressed in lens primary cultures. Seven days after the infection, primary lens cultures infected by retroviruses, RCAS(A) (a, e), -Cx45.6 (b, c), and -Cx56 (f, g) were fixed and labeled with anti-Cx45.6 (a, b), anti-Cx56 (e, f) or anti-FLAG antibody (c, g). The primary antibodies were detected by fluorescein-conjugated anti-rabbit IgG for anti-Cx45.6 and Cx56, and rhodamine-conjugated anti-mouse IgG for anti-FLAG antibody. The immunostaining was visualized by confocal fluorescence microscope. The corresponding merged images derived from (b) and (c), and (e) and (f) are shown in (d) and (h), respectively. Bar, 80 μm .

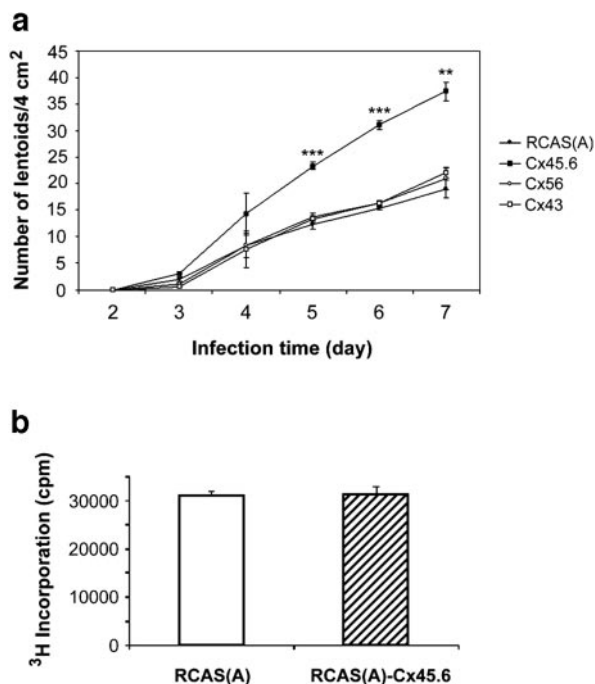


FIGURE 4. Overexpression of Cx45.6, but not of Cx43 and Cx56, stimulated the formation of lentoid structures in lens primary cultures, but did not affect lens cell proliferation. (a) Primary cultures infected with the retroviruses RCAS(A) (vehicle) or RCAS(A)-Cx43, -Cx45.6, or -Cx56 at the second day of cell plating were examined, and the total number of lentoids formed was quantified for various culture periods (***P* < 0.01; ****P* < 0.001). The data are presented as mean ± SD (*n* = 5). (b) The rate of lens cell proliferation was measured in RCAS(A) or RCAS(A)-Cx45.6-infected cultures by [³H]thymidine incorporation assay. The data are presented as the mean ± SD (*n* = 3).

were examined (Figs. 5, 6). MIP, a member of the aquaporin family also called AQP0, is expressed only in the differentiating lens fiber cells at a relatively later developmental stage than other differentiation markers.³⁶ We found that lentoids formed in Cx45.6-overexpressing cultures express MIP the same as those formed under normal conditions and in Cx56-overexpressing cells (Fig. 5a). On the second day after cell seeding, retroviruses expressing Cx45.6, Cx56, and RCAS(A) vehicle were added to separate lens primary culture dishes and incubated for up to 6 days. At the last day of culture, MIP protein was labeled using monoclonal antibody specific for MIP. The

size of MIP-positive immunofluorescence stained areas in the connexin-overexpressing or control cultures was measured and quantified. Similar to the data from the quantification of lentoids shown in Figure 4a, the cultures overexpressing Cx45.6 had close to a onefold increase in MIP expression compared with Cx56-overexpressing or RCAS(A) vehicle control cultures (Fig. 5b). Compared with lens culture infected with retrovirus, RCAS(A), the total amount of δ-crystallin molecules in Cx45.6-overexpressing cultures also increased (Fig. 6). From the time period examined, the level of δ-crystallin expression was elevated on the second day after infection and continued until 8 days, whereas the amount of β-actin remained relatively constant during the same period (Fig. 6a). The increased δ-crystallin protein level compared with controls was further confirmed by densitometric measurement (Fig. 6b).

Together, these results show that the overexpression of Cx45.6 promotes lens epithelial-fiber cell differentiation by the increased expression of the lens differentiation marker proteins, MIP and δ-crystallin.

Stimulatory Effect of Cx45.6 on Differentiation

To determine whether the stimulation of lentoid formation by overexpression of Cx45.6 is due to an alteration of functional gap junctions, two types of dye-transfer approaches were adopted to assess intercellular coupling: single-cell microinjection and scrape-loading dye transfer. In addition, two different types of tracer molecules, LY and biocytin, were used (Figs. 7, 8). Microinjection analysis shows that, within the identical period, the distance that LY traveled beyond injected cell was similar between the control and Cx45.6-overexpressing cells (117.84 ± 16.54 μm vs. 119.60 ± 16.52 μm; *n* = 3; Fig. 7a). The scrape-loading dye-transfer assay also revealed no significant alteration in intercellular coupling of LY (Fig. 7b).

To further verify the results from LY dye-transfer analysis, we took advantage of another commonly used tracer molecule, biocytin, to explore the activities of intercellular coupling. Because of its lower molecular mass, ionic charge, and/or more sensitive methods of detection (fluorochrome-labeled avidin), biocytin has been shown to be more permeable to gap junction channels than LY.^{17,37} No difference in the extent of dye-transfer was observed between Cx45.6-overexpressing and RCAS(A) vehicle-infected lens cultures (Figs. 8c, 8f).

The results obtained from dye-transfer studies using two types of tracer molecules and two different dye-transfer approaches suggest that the stimulatory effect of lens cell differentiation by Cx45.6 are independent of intercellular coupling.

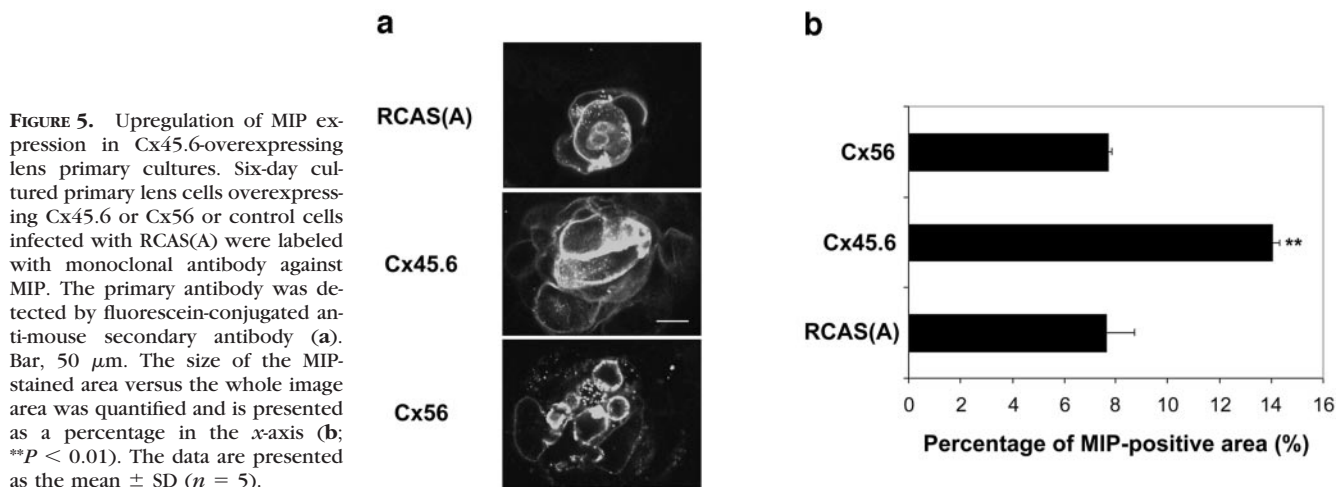


FIGURE 5. Upregulation of MIP expression in Cx45.6-overexpressing lens primary cultures. Six-day cultured primary lens cells overexpressing Cx45.6 or Cx56 or control cells infected with RCAS(A) were labeled with monoclonal antibody against MIP. The primary antibody was detected by fluorescein-conjugated anti-mouse secondary antibody (a). Bar, 50 μm. The size of the MIP-stained area versus the whole image area was quantified and is presented as a percentage in the x-axis (b; ***P* < 0.01). The data are presented as the mean ± SD (*n* = 5).

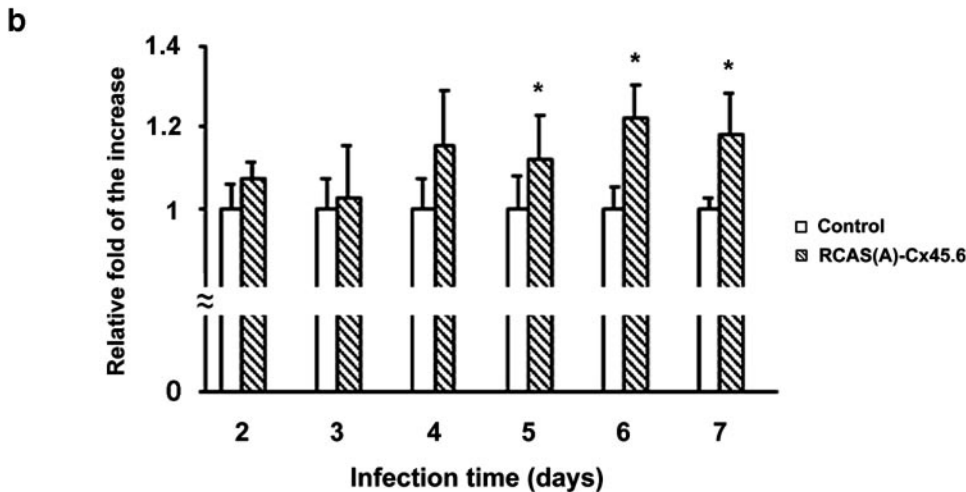
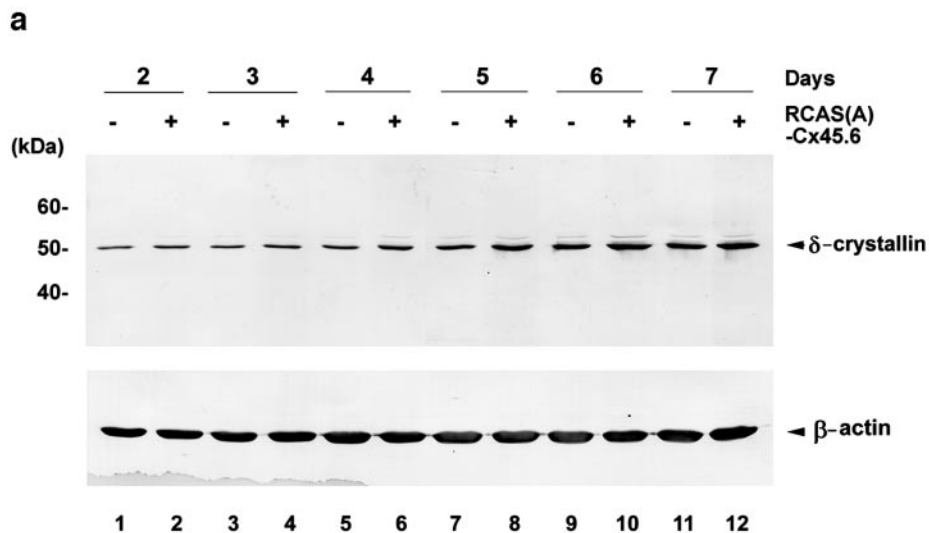


FIGURE 6. δ-Crystallin was upregulated in Cx45.6-overexpressing lens primary cultures. **(a)** Lens primary cultures were infected with high-titer retroviruses RCAS(A) (lanes 1, 3, 5, 7, 9, and 11) or RCAS(A)-Cx45.6 (lanes 2, 4, 6, 8, 10, and 12) at the second day after cell plating and were cultured for an additional 2 (lanes 1 and 2), 3 (lanes 3 and 4), 4 (lanes 5 and 6), 5 (lanes 7 and 8), 6 (lanes 9 and 10), and 7 (lanes 11 and 12) days. The cell lysate was loaded on an SDS-polyacrylamide gel and immunoblotted with anti-δ-crystallin and β-actin antibodies. **(b)** The δ-crystallin bands from three separate Western blot analysis were quantified by densitometry. The relative magnitudes of increase compared with RCAS(A) control cultures are presented on the y-axis. The data are expressed as the mean ± SD (*n* = 3). (**P* < 0.05 compared with the non-Cx45.6-overexpressing control at the same time point).

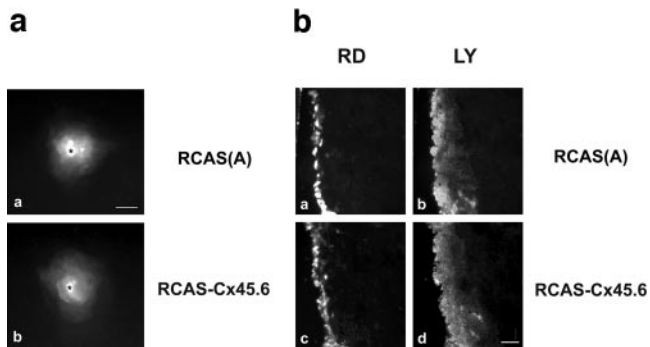


FIGURE 7. The overexpression of Cx45.6 did not alter the intercellular LY dye coupling in lens primary cells. The second day after cell plating, lens primary cultures were infected with high-titer retroviruses, RCAS(A) (vehicle, **aa**, **ba**, **bb**) or RCAS(A)-Cx45.6 (**ab**, **bc**, **bd**) and were further cultured for 3 days. **(a)** The lens epithelial cells (*) were microinjected with LY, and the image was taken 5 minutes after injection (*n* = 3). Bar, 50 μm. **(b)** The scrape-loading dye transfer assay was performed using RD (**ba**, **bc**) as a tracer dye and LY (**bb**, **bd**) as a transferring dye (*n* = 3). Bar, 35 μm.

Effect of Cx45.6 on Expression of Endogenous Connexins

To examine whether the overexpression of Cx45.6 has any effect on endogenous connexin expression, infection with the retrovirus RCAS(A)-Cx45.6 was initiated at various time during cell plating (i.e., at 2 or 3 days) The expression of connexins was uniformly examined after 8 days of culture. The protein levels of connexins were analyzed by immunoblots (Fig. 9; *n* = 3), whereby exogenous Cx45.6 was detected with epitope tag anti-FLAG antibody (Fig. 9a), and endogenous Cx43 (Fig. 9b), Cx45.6 (Fig. 9c), and Cx56 (Fig. 9d) were detected by their corresponding antibodies. We found that the longer the infection time, the more exogenous Cx45.6 was expressed. In addition, we found that the more the cells expressed exogenous Cx45.6, the more dramatic the effect on stimulation of lens differentiation (data not shown). Exogenous Cx45.6 expression had no direct effect on the level of endogenous Cx43 (Fig. 9b) and Cx56 (Fig. 9d) expression. Blots by anti-Cx45.6 antibody revealed both endogenous and exogenous Cx45.6 expression (Fig. 9c). The increased Cx45.6 levels associated with infection time were indicative of increased exogenous Cx45.6 expression, a result consistent with that detected by the FLAG antibody (Fig. 9a). The Western blot results were

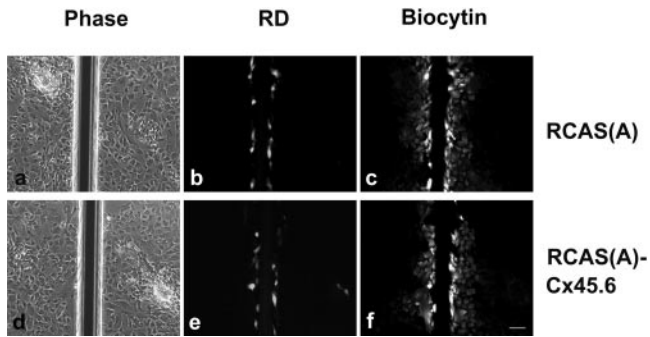


FIGURE 8. Biocytin dye coupling was not affected by the overexpression of Cx45.6 in lens primary cells. The second day after cell seeding, lens primary cultures were infected with high-titer retroviruses, RCAS(A) (vehicle, **a-c**) or RCAS(A)-Cx45.6 (**d-f**) and were further cultured for 3 days. The scrape-loading dye transfer experiments were performed with RD (**b, e**) as a loading tracer dye and biocytin (**c, f**) as a transferring dye. The transfer of biocytin was detected by avidin-fluorescein. Phase images are presented in (**a**) and (**d**) ($n = 3$). Bar, 35 μm .

confirmed by a densitometric measurement of the intensity of the bands (bottom panel of each blot).

Together, our results suggest that exogenously expressed connexins are colocalized with their endogenous counterparts and the stimulatory effect on lens cell differentiation is mediated exclusively by the overexpression of Cx45.6, but not by the altered expression of other lens connexins.

DISCUSSION

In this study, we showed the efficient expression of exogenous lens connexins in primary cultures of lens cells by using retroviruses containing corresponding recombinant connexin constructs, a technique we had adopted to express exogenous lens connexins in lens *in vivo*.²⁵ This approach has been shown to be highly efficient. We found that after a few days of retroviral infection, almost all the primary cultured cells expressed epitope-tagged exogenous connexins. Exogenous lens fiber connexins Cx45.6 and Cx56 were predominantly localized in lentoid bodies and to a much lesser extent in lens epithelial

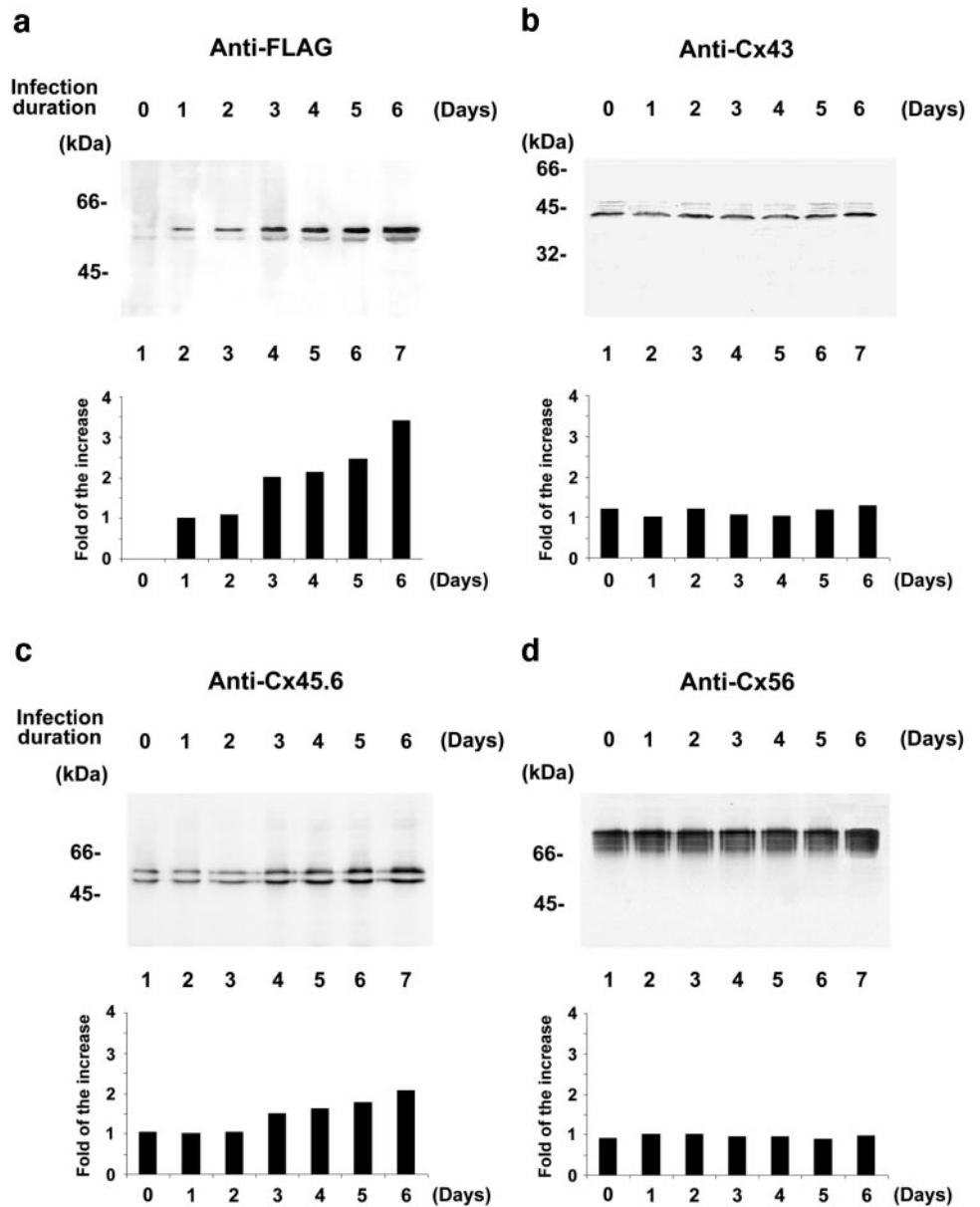


FIGURE 9. Exogenous Cx45.6 expression did not affect the protein levels of endogenous lens connexins. After the plating of lens primary cultures, these cultures were infected with the retrovirus Cx45.6 at designated time points during cell plating: days 1 (lane 2), 2 (lane 3), 3 (lane 4), 4 (lane 5), 5 (lane 6), 6 (lane 7), and noninfected (lane 1), and cells were all collected at day 8. These resulted in the total duration of retroviral infection of time 0 (lane 1) and days 1 (lane 2), 2 (lane 3), 3 (lane 4), 4 (lane 5), 5 (lane 6), and 6 (lane 7). Western blot analyses were performed with anti-FLAG (**a**), anti-Cx43 (**b**), anti-Cx45.6 (**c**), and anti-Cx56 (**d**) antibodies. The connexin bands from Western blot experiments were quantified by densitometric measurements and presented underneath each blot. The y-axis represents the magnitude of the increase relative to the protein levels after 1 day of the retroviral infection ($n = 3$).

cells. This observation is consistent with the expression pattern of their endogenous counterparts in primary lens cell cultures¹⁷ as well as with the moderate expression pattern of these two connexins throughout the anterior epithelium of embryonic chick lens in vivo.³⁸ Moreover, we found that exogenous Cx43 was detectable in the edges and surface areas of lentoid bodies, in addition to the expression in epithelial cells, which concurs with the observation of endogenous Cx43 expression in lens primary cultures.¹⁷

In this study, we have demonstrated that the overexpression of Cx45.6 stimulated lens cell differentiation and induced early formation of lentoid bodies. The lentoids formed from Cx45.6-overexpressing cultures were found to be similar to those of the normal cell differentiation processes, yielding high expression levels of the lens fiber markers, such as MIP and δ -crystallin. Previous studies have shown that mice without either of the fiber connexins Cx46 or Cx50 show development of lens cataract^{7,8}; however, only Cx50-deficient postnatal mice have smaller eyes, defined as microphthalmia,⁸ and exhibit a delayed lens fiber maturation.⁹ The postnatal occurrence of lens growth retardation and the delayed fiber maturation indicate that the formation of mature secondary fibers, a process that continues throughout life, is likely to be affected. The appearance of lentoid bodies in the embryonic lens primary culture resembles the process of secondary fiber formation in lens in situ. At the embryonic developmental stage when the lens cells are isolated for culturing, the stem epithelial cells from the lens equator continue to differentiate and form fiberlike lentoid structures. Therefore, this embryonic primary lens culture system provides a unique opportunity to study the molecular mechanism behind epithelial cell differentiation and formation of mature secondary fibers. Studies by Yang and Louis³⁹ show that in contrast to the level of Cx44, the ovine orthologue of Cx56, which remains low, the level of Cx49, the ovine orthologue of Cx45.6, is increased in association with the differentiation of lens primary culture. This result suggests that Cx49 has a unique temporal pattern of expression during lens cell differentiation, which corresponds in part to our observation of the unique function of chick Cx45.6 in lens epithelial-fiber cell differentiation. Therefore, our results combined with other previous observations suggest that expression of Cx45.6 is important in lens epithelial cell differentiation and formation of mature secondary fibers.

We have observed that stimulation of lens cell differentiation by Cx45.6 appears not to be directly correlated with obvious alteration in intercellular coupling. To validate the dye-transfer analyses, we used two dye-transfer approaches: microinjection and scrape-loading. In addition, we used two types of tracer molecules, LY and biocytin. Using these approaches, we have previously observed the alterations of dye-transfer levels in responding to other regulatory factors.⁴⁰ Consistent with our observation, studies by Le and Musil¹⁷ show that the inhibition of intercellular couplings with the gap junction blocker, 18 β -glycyrrhetic acid, has no effect on lens epithelial-fiber cell differentiation and lentoid formation in primary lens cells. This observation is at odds with an earlier report that the inhibition of gap junction communication by Rous sarcoma virus blocks fiber cell differentiation.²⁴ In these inhibitory studies, general channel inhibitors of gap junctions were used instead of specific reagents. Thus, it is difficult to evaluate the inhibitory effect on activities of individual connexins. One possible interpretation for this controversy is that although the inhibitors used can block the activities of certain types of gap junction channels detected by dye-transfer assay, the channels formed by other connexins such as Cx45.6 may not be affected by the same inhibitor. Another distal possibility is that Cx45.6 may not fully participate in the formation of functional gap-junction-mediated intercellular channels in the

lens epithelial cells. Therefore, the existence of uncharacterized functional properties of Cx45.6 cannot be excluded at this point. In other cellular systems, the existence of functional hemichannels formed by connexins at non-gap-junction regions of the cell has been reported.⁴¹ A couple of studies have also shown that Cx50 forms hemichannels in *Xenopus* oocytes and transfected cells.^{42,43} The function and regulatory mechanism of these hemichannels in lens cells are yet to be elucidated.

We have shown that exogenous connexins are mostly colocalized with their endogenous counterparts, suggesting that they are likely to behave in a fashion similar to endogenous connexins. Endogenous Cx45.6 and Cx56 are colocalized throughout the embryonic lenses and form heteromeric connexons in the lens.¹⁸ The electrical conductance properties of these heteromeric connexons are different from channels formed with either of the lens connexins.⁴⁴ Despite their identical localization and coexistence in the same connexons, we have determined that only Cx45.6 promoted lens cell differentiation. Previous reports have shown that the orthologues of Cx45.6 and Cx56 display differential physiological and gating properties. The Cx45.6 orthologue displays a high sensitivity in pH gating and appears to regulate the Cx56 orthologue in differentiating fiber,⁴⁵⁻⁴⁷ whereas the Cx56 orthologue exhibits less pH sensitivity and is responsible for coupling mature fibers.^{46,47} These physiological differences could be accounted for partially by the uniqueness of the C-terminal sequences between connexins, which makes it likely that certain regulatory factor(s) bind to that region of Cx45.6 using a lesser-known mechanism to initiate the process leading to the formation of lens secondary fibers. Direct interaction between C-terminal domains of gap junction protein Cx43 or Cx45 with ZO-1 has been observed. This association may serve to localize Cx43 to certain regions in cells.⁴⁸ Recently, Cx43 is reported to interact directly with tubulin, suggesting that in addition to its role as a channel-forming protein, Cx43 may influence the properties of microtubules in cells.⁴⁹ The possibility of interactions of Cx45.6 with other potential factor(s) that facilitate roles of Cx45.6 in the formation of lens fibers cannot be excluded at this stage.

Our study has revealed a novel function and mechanistic aspects of lens Cx45.6 in lens epithelial-fiber cell differentiation and formation of lens fibers, which serves as a starting point directed toward the further understanding of the molecular mechanism and regulation underlying in vivo cell differentiation processes facilitated by Cx45.6.

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