# Promotion of lens epithelial-fiber differentiation by the C-terminus of connexin 45.6 – a role independent of gap junction communication

**Eric A. Banks, X. Sean Yu, Qian Shi and Jean X. Jiang***

Department of Biochemistry, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA
*Author for correspondence (e-mail: jiangj@uthscsa.edu)

*Accepted 6 August 2007*
*Journal of Cell Science 120, 3602-3612 Published by The Company of Biologists 2007*
*doi:10.1242/jcs.000935*

## Summary

**We previously reported that, among the three connexins expressed in chick lens, overexpression of connexin (Cx) 45.6, not Cx43 or Cx56, stimulates lens cell differentiation; however, the underlying mechanism responsible for this effect is unclear. Here, we took advantage of naturally occurring loss-of-gap-junction function mutations of Cx50 (ortholog of chick Cx45.6) and generated the corresponding site mutants in Cx45.6: Cx45.6(D47A) and Cx45.6(P88S). In contrast to wild-type Cx45.6, the mutants failed to form functional gap junctions, and Cx45.6(P88S) and, to a lesser degree, Cx45.6(D47A) functioned in a dominant-negative manner. Interestingly, overexpression of both mutants incapable of forming gap junctions significantly increased epithelial-fiber differentiation to a level comparable to that of wild-type Cx45.6. To map the functional domain of Cx45.6, we generated a C-terminus chimera as well as deletion mutants. Overexpression of Cx56*45.6C, the mutant in which the C-terminus of Cx56 was replaced with that of Cx45.6, had a stimulatory effect on lens cell differentiation similar to that of Cx45.6. However, cells overexpressing Cx45.6*56C, the mutant in which C-terminus of Cx45.6 was replaced with that of Cx56, and Cx45.6(–C), in which the C-terminus was deleted, failed to promote differentiation. Taken together, we conclude that the expression of Cx45.6, but not Cx45.6-dependent gap junction channels, is involved in lens epithelial-fiber cell differentiation, and the C-terminal domain of Cx45.6 plays a predominant role in mediating this process.**

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/20/3602/DC1

Key words: Connexin 45.6, Mutants, Gap junctions, Cell communication, Lens cell differentiation

## Introduction

Gap junctions are well-defined clusters of transmembrane channels connecting the cytoplasm of adjacent cells, which allow molecules (molecular mass ≤1 kDa), such as metabolites, ions and second messengers, to pass through. This type of cell-cell communication is crucial in maintaining normal cell and tissue functions (Goodenough et al., 1996). The eye lens, suspended from the ciliary body, retains a stem cell population that continues to proliferate and differentiate throughout the life of an organism (Bloemendal, 1977; McAvoy et al., 1999). Epithelial cells covering the anterior lens surface fold posteriorly at the equator, where they are continuous during both lens fiber differentiation and formation. The fibers at the center of the lens are coupled with cells at the lens surface through a highly developed intercellular communication network mediated by gap junctions. This extensive network is vital as it facilitates the exchange of ions and metabolites throughout the avascular lens, maintaining osmotic and metabolic homeostasis and lens transparency (Goodenough, 1992; Mathias et al., 1997). The monomeric proteins from which gap junctions are assembled are members of the connexin family and consist of four conserved transmembrane domains and two conserved extracellular loop domains. The sequences of the cytoplasmic domain, particularly at the C-terminus, are diverse. Three connexins have been identified in the mammalian lens, Cx43, Cx46 and Cx50, of which Cx43 is specific to lens epithelial cells, whereas Cx46 and Cx50 predominantly colocalize in lens fibers. Previous studies have shown that mice lacking genes encoding either fiber connexin Cx46 or Cx50 develop lens cataracts (Gong et al., 1997; White et al., 1998; Rong et al., 2002); however, only Cx50-deficient mice develop smaller eyes defined as microphthalmia (White et al., 1998; Rong et al., 2002) and exhibit delayed maturation of lens fibers (Rong et al., 2002). Replacement of Cx50 with Cx46 by genetic knock-in prevents cataracts, but does not restore normal growth of the lens (White, 2002). Moreover, genetic studies show that expression of Cx50 appears to be required for development of lenses of normal size in mice (Martinez-Wittinghan et al., 2003). The postnatal occurrence of lens growth retardation and delayed fiber maturation indicate that the formation of mature secondary fibers is likely to be affected.

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 (Menko et al., 1984; Berthoud et al., 1999). Monolayer lens epithelial cells gradually differentiate into structures called lentoids. The appearance

of lentoid bodies in embryonic lens primary culture resembles the process of lens secondary fiber formation in situ, providing a unique opportunity to explore the molecular mechanism underlying epithelial-fiber cell differentiation and formation of mature secondary fibers. Assessing lentoid formation can closely follow the process of lens epithelial-fiber cell differentiation associated with expression of lens differentiation markers such as the cytoskeletal proteins filensin and CP49 (Blankenship et al., 2001). Furthermore, lentoids express a differentiation marker major intrinsic protein (MIP), also called aquaporin-0 (AQP0), which is expressed primarily in mature fibers during lens development (Yu and Jiang, 2004). However, in other species, such as rodent or cattle, lens primary cultures only partially differentiate, as evidenced by incomplete phosphorylation of connexins in comparison with the levels of in vivo phosphorylation (Jiang et al., 1993). Like mammalian lens, the chick lens contains three connexins: Cx43, Cx45.6 and Cx56 (Musil et al., 1990; Jiang et al., 1994; Rup et al., 1993), the orthologs of mammalian Cx43, Cx50 and Cx46, respectively. Cx45.6 and Cx56 are expressed predominately in fiber cells in lens organs (Jiang et al., 1995; Dahm, 1999) and in lens primary cultures (Le and Musil, 1998). Cx45.6 and Cx56 colocalize and form heteromeric hemichannels that are also called heteromeric connexons (Jiang and Goodenough, 1996).

Several mutations of genes encoding Cx46 and Cx50 have been identified that are directly linked to human autosomal congenital cataracts and cataract formation in mice (Shiels et al., 1998; Berry et al., 1999; Steele, Jr et al., 1998; Mackay et al., 1999; Rees et al., 2000; Pal et al., 1999; Gerido and White, 2004). Among these mutations, mouse Cx50 mutant D47A, located at the first extracellular loop domain, was characterized as a loss-of-function mutation, unable to form functional gap junction channels (Steele, Jr et al., 1998; Xu and Ebihara, 1999). Cx50 missense mutant P88S at the second transmembrane domain, associated with human autosomal dominant-negative cataracts, has been reported not only to fail to form functional gap junctions but also to act as a dominant-negative mutant that inhibits wild-type connexins from forming gap junctions (Shiels et al., 1998; Pal et al., 1999). We previously have shown that one of the lens connexins, Cx45.6, unlike the other two types of lens connexins, stimulates epithelial-fiber cell differentiation and expression of major differentiation markers (Gu et al., 2003). Moreover, this stimulatory effect appears to be independent of lens cell proliferation and intercellular coupling. However, no previous studies have demonstrated that the unique role of Cx45.6 in lens differentiation is independent of its conventional role in intercellular communication.

To investigate the novel function of Cx45.6, we used two particular Cx45.6 mutants because their respective mammalian counterparts are directly linked to formation of functionally impaired gap junction channels. In this study, we show that, although these mutants fail to form functional gap junction channels, they promote epithelial-fiber differentiation to a level comparable to that of wild-type Cx45.6. Our data demonstrate that the function of Cx45.6 in lens cell differentiation is independent of its role in forming functional gap junctions. Furthermore, the C-terminal domain of Cx45.6 is functionally involved in this action.

## Results

### Retroviral expression and cellular distribution of exogenous Cx45.6 and mutants

The Cx45.6 mutation D47A is located on the first extracellular loop domain, whereas mutation P88S is located within the second transmembrane region. The exogenous overexpression of wild-type Cx45.6 and its mutants was achieved by cell infection with recombinant retroviruses containing the appropriate DNA constructs. To achieve expression of wild-type and mutant Cx45.6, we used a lens connexin-null chicken embryonic fibroblast (CEF) cell line. Western blot analysis showed the expression in lens connexin-null CEF cells of wild-type Cx45.6 (Fig. 1A, lane 3) and the mutants Cx45.6(D47A) (lane 1) and P88S (lane 2). Similar to the expression pattern of wild-type Cx45.6 we reported previously (Gu et al., 2003) and Cx56 (Berthoud et al., 1999), high-resolution immunofluorescence confocal microscope experiments with antibodies against the FLAG epitope tag and against Cx45.6 revealed in primary cultured lens cells that the Cx45.6 mutants distributed primarily to the lentoid structures. The staining patterns with sharp outlines indicate a membrane localization for Cx45.6 and the corresponding mutants (Fig. 1B). Intriguingly, exogenously expressed wild-type Cx45.6 and its mutants labeled by FLAG staining reveal some intracellular signals that are not shown when labeling with the antibody against Cx45.6, which detects both endogenous and exogenous connexins. One possible explanation is that the antibody against the FLAG tag that works best in our system generates higher background fluorescence in comparison with that from the Cx45.6 antibody we ourselves generated and affinity purified. Alternatively, but less likely, the antibody against FLAG is somehow more accessible to intracellularly expressed connexins in comparison with the antibody against Cx45.6. Together, these results suggest that the retroviral approach efficiently expresses exogenous Cx45.6 and mutants thereof. The expression and distribution of exogenous connexins are not affected by the respective site mutations. The images presented offer a novel structural view and strengthen the use of lentoids as an in vitro model for lens differentiation.

### Intercellular communication mediated by wild-type, but not mutant, Cx45.6

Cx45.6 mutations of the corresponding amino acid residues D47A and P88S have been reported as loss-of-gap-junction-function mutations of Cx50 based on examinations in a *Xenopus* oocyte expression system (Xu and Ebihara, 1999; Berry et al., 1999; Pal et al., 1999), and P88S also functions in a dominant-negative manner (Xu and Ebihara, 1999). To determine whether these Cx45.6 mutants form functional gap junction channels in cells, wild-type and mutant Cx45.6 were expressed in CEF cells through retroviral expression. Two types of commonly used dye-transfer approaches were used to assess intercellular coupling: invasive scrape-loading dye transfer and non-invasive parachuting dye transfer. In addition, three different types of tracer molecules, LY, Alexa 488 and calcein, were used (Fig. 2). As we reported previously, with retroviral infection, almost all cells express exogenous connexins (Jiang, 2001; Gu et al., 2003). In addition, CEF cells do not express native Cx45.6. The extent of dye transfer was measured by the distance from the scrape line to the cell front where dye transfer stops. Alexa 488 and LY were measured

from the scrape and rhodamine dextran (RD) served as a non-transfering control. Cells initially receiving both dyes indicate entry points, whereas cells with Alexa 488/LY alone show the extent of gap junction intercellular communication. CEF cells expressing exogenous wild-type Cx45.6 show an almost 100% increase in Alexa 488 transfer over cells expressing mutant connexins and the control (Fig. 2A). LY transfer is >100% greater than that of mutant proteins and the control, although the transfer by RCAS(A) virus containing the empty vector control (vehicle) appears to be slightly higher than Alexa 488 (Fig. 2B). No significant difference was observed among the respective mutants versus control for either dye. The extent of RD staining was about the same (approximately 25 μm) for both Alexa 488 and LY experiments. Some low levels of coupling were observed by the vehicle controls in CEF cells, which could be attributed to the low expression levels of other connexins, although we could not detect the expression of several connexin proteins, including Cx43, Cx45.6, Cx56, Cx42, Cx32 and Cx26, by western blots. To further confirm the loss-of-function of these mutants, the non-invasive parachuting dye-transfer method was used. CEF cells transfected with mutant and wild-type connexins plus vector control were pre-loaded with calcein acetoxymethyl ester (AM) and dialkylcarbocyanine dye (DiI). Pre-loaded cells were layered over their respective adherent cell cultures. Immunofluorescence microscopy with merged images shows how the dye transfer emanates from pre-loaded cells (Fig. 2C). Cells expressing wild-type Cx45.6 show a 100% transfer increase over vehicle (control) and mutants as measured by the calcein dye-transfer area. There was no difference among mutants and controls (Fig. 2C, lower panel). DiI served as a pre-loaded cell membrane marker and cannot be transferred. In addition, Cx50(P88S) has been reported not only to fail to form functional channels but also to function in a dominant-negative manner (Pal et al., 1999). To probe whether the corresponding Cx45.6 mutant could attenuate the role of wild-type Cx45.6 in forming gap junctions, wild-type Cx45.6 along with Cx45.6(P88S) and Cx45.6(D47A) were coexpressed in CEF cells, and scrape loading (Fig. 3A) and parachuting (Fig. 3B) dye-transfer assays were then conducted. The dye transfer mediated by wild-type Cx45.6 was almost completely attenuated by Cx45.6(P88S) and was abrogated to a slightly lesser degree by Cx45.6(D47A). Together, these results suggest that, unlike wild-type Cx45.6, both mutants fail to form functional gap junction channels in the cell. In addition, Cx45.6(P88S) and Cx45.6(D47A) behave as dominant-negative mutants, blocking the ability of wild-type Cx45.6 to form gap junctions.

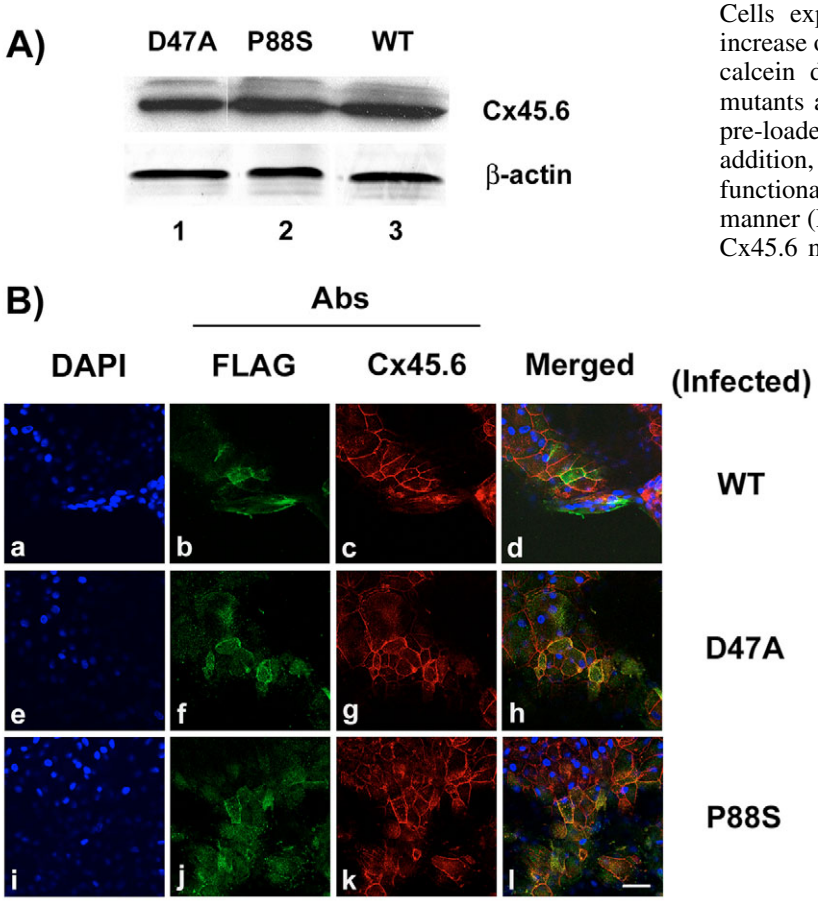


**Fig. 1.** Expression and co-distribution of wild-type and mutant Cx45.6. (A) Recombinant retroviruses containing cDNAs encoding wild-type Cx45.6 (lane 3) and mutant Cx45.6(D47A) (lane 1) and Cx45.6(P88S) (lane 2) proteins were used to infect CEF cells. After 6 days of infection, cells were lysed, and crude membranes were prepared and analyzed by SDS-PAGE. Western blots were performed by probing PVDF replicas with affinity-purified antibody recognizing the C-terminus of Cx45.6. The membrane was stripped and re-probed with monoclonal antibody against the control protein, β-actin. (B) Eight days after infection of recombinant retroviruses, primary cultured cells expressing exogenous wild-type Cx45.6 (a-c), mutant proteins Cx45.6(D47A) (e-g) and Cx45.6(P88S) (i-k) were fixed, stained with DAPI (a,e,i) and labeled with antibody against FLAG (b,f,j) or against Cx45.6 (c,g,k). The primary antibodies were detected by fluorescein-conjugated anti-mouse IgG for the antibody against FLAG and rhodamine-conjugated anti-rabbit IgG for the antibody against Cx45.6. The immunostaining was visualized by confocal fluorescence microscopy. The corresponding merged images derived from a,b,c, e,f,g and i,j,k are shown in d, h and l, respectively. Bar, 10 μm.

### Loss-of-gap-junction-function mutants of Cx45.6 stimulate epithelial-fiber differentiation to a degree similar to that of wild-type Cx45.6

We have previously shown that overexpression of wild-type Cx45.6 induces an ~100% increase in the amount of MIP(AQP0) over wild-type Cx45.6 (Gu et al., 2003). Compared with protein levels of vehicle and β-actin controls, western blots of membrane preparations with antibody against Cx45.6 and densitometric measurements revealed the increased expression of Cx45.6 and mutants in all connexin-overexpressing cultures (Fig. 4A). Consistent with our previous report, some phosphorylated Cx45.6 was also present (lower migrating band) (Jiang et al., 1994; Jiang and Goodenough, 1998; Yin et al., 2001). We used MIP(AQP0), a defined differentiation marker (Zhou et al., 2002; Yu and Jiang, 2004), for quantitatively evaluating the extent of transition from epithelial to fiber cell in chicken lens primary

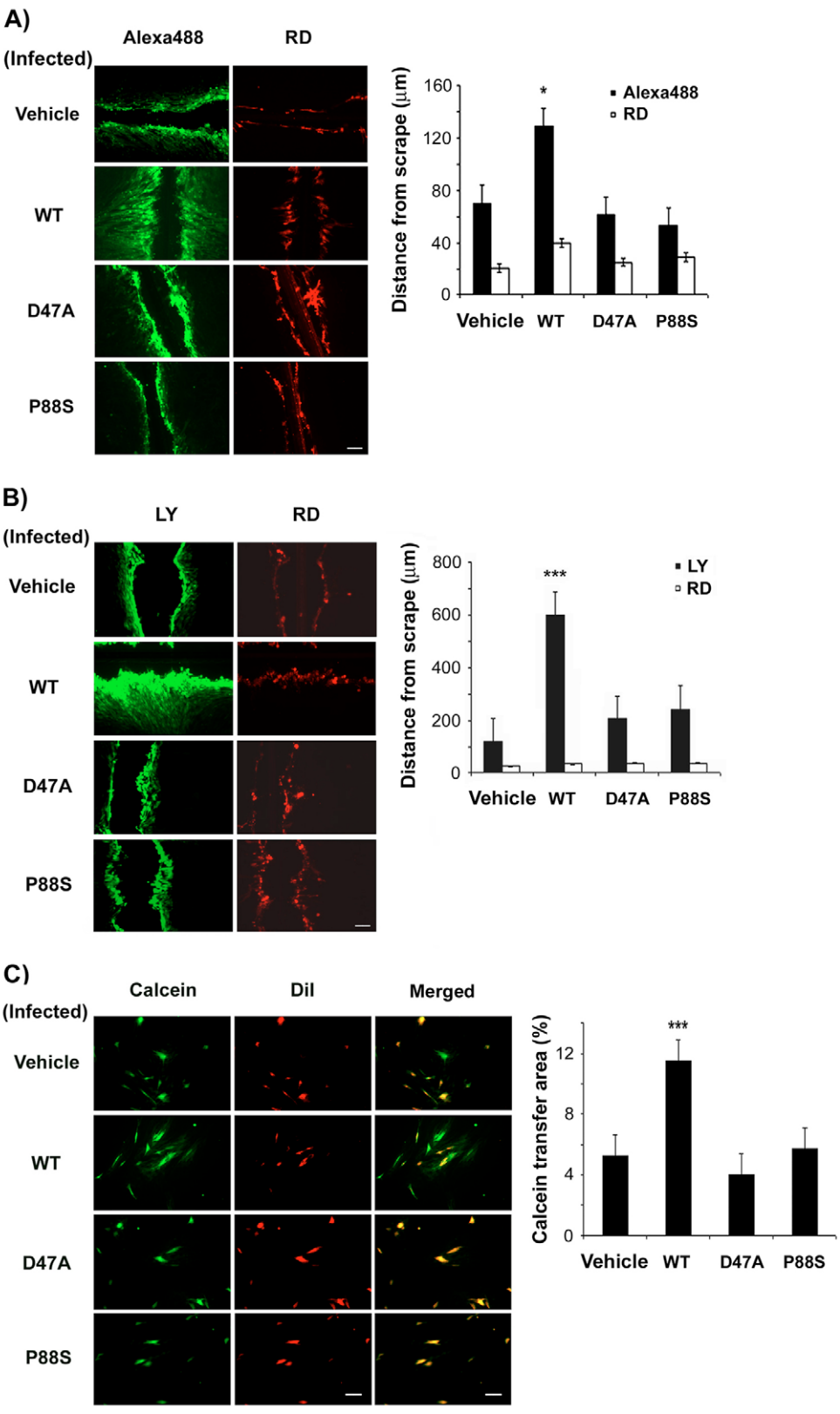


**Fig. 2.** Wild-type, not Cx45.6 mutant, proteins form functional gap junction channels that mediate dye transfer. (A,B) Six days after retroviral infection with recombinant retroviruses RCAS(A)-Cx45.6, RCAS(A)-Cx45.6(D47A) and RCAS(A)-Cx45.6(P88S), the scrape-loading dye-transfer assay was performed using RD as a tracer dye (A,B) and Alexa488 (A) or LY (B) as transferring dyes. Bar, 40 μm. The extent of dye transfer was quantified by measuring the distance from scrape lines to the migrated front of cells stained with Alexa 488 (A, lower panel) or LY (B, lower panel). The data are presented as the mean±s.e.m.; $n$=5. (*$P$<0.05; ***$P$<0.001, in comparison with the non-Cx45.6-overexpressing control). Bar, 10 μm. (C) Six days after retroviral infection with recombinant retroviruses RCAS(A)-Cx45.6, RCAS(A)-Cx45.6(D47A) and RCAS(A)-Cx45.6(P88S), a parachuting dye-transfer assay was performed using DiI as a tracer dye and calcein as a transferring dye. The extent of dye transfer was quantified by measuring the area of calcein-fluorescence-stained cells (NIH image; lower panel). The data are presented as the mean±s.e.m.; $n$=3. ***$P$<0.001, in comparison with the non-Cx45.6-overexpressing control. Bar, 10 μm.

culture. Overexpression of Cx45.6 induced a 100% increase in the level of MIP(AQP0) over that of a vehicle control (Fig. 4B, lower panel). More importantly, we show a similar 100% MIP(AQP0) increase induced by overexpression of the two mutants, D47A and P88S (Fig. 4B, lower panel). Furthermore, our results show no significant difference among the mutants in comparison with the wild-type protein (Fig. 4B, lower panel). To further evaluate MIP(AQP0) expression in primary

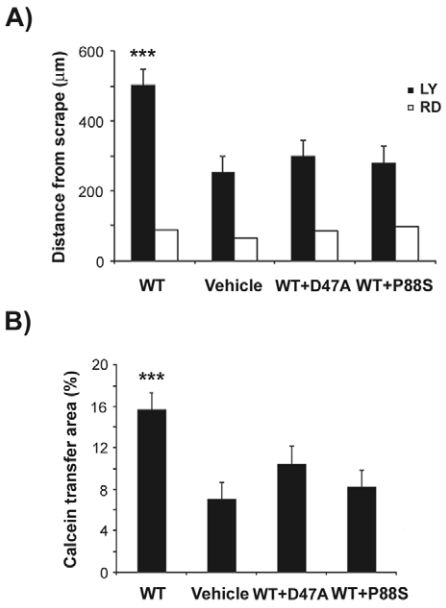


**Fig. 3.** Mutant proteins Cx45.6(P88S) and Cx45.6(D47A) inhibit the intercellular coupling mediated by wild-type Cx45.6. CEF cells were co-infected with recombinant retroviruses RCAS-Cx45.6 and mutants RCAS(A)-Cx45.6(P88S) and RCAS(A)-Cx45.6(D47A). The extent of dye transfer was quantified by the scrape-loading dye-transfer assay using LY-RD (A) and parachuting dye-transfer assay using calcein-Dil (B). The data are presented as mean±s.e.m.; $n$=3. ***$P$<0.001, in comparison with the non-Cx45.6-overexpressing control.

lens cultures, adherent primary cells were immunolabeled for MIP(AQP0), and the MIP(AQP0)-positive stained area was then analyzed and quantified (Fig. 4C). As expected, MIP(AQP0) was expressed mainly in the lentoid-containing region (Fig. 4C, phase-contrast versus fluorescence images). We show a similar but nearly 100% increase in MIP(AQP0) expression by MIP(AQP0)-positive area quantification. This observation is comparable to the ~100% increase of MIP(AQP0) assessed by western blot analysis. Again, no significant difference among the mutant and wild-type proteins was observed (Fig. 4C, right panel). To further determine the extent of lens epithelial-fiber differentiation, we counted the numbers of lentoid structures formed in cells when overexpressing wild-type Cx45.6 and mutants of Cx45.6. Consistently, compared with vehicle controls, there is a 100% increase in the numbers of lentoids in Cx45.6 and mutant-overexpressing lens cells measured eight days post infection (Fig. 5A). Furthermore, overexpression of Cx45.6 and the mutant proteins also increased the protein levels of two known lens differentiation markers, filensin and CP49 (Fig. 5B) (Blankenship et al., 2001). Together, these results show that Cx45.6 mutants, although they cannot form functional gap junction channels, promote epithelial-fiber differentiation in a manner similar to that of wild-type Cx45.6. More importantly, dominant-negative Cx45.6 mutants that would block the endogenous gap junction channels formed by Cx45.6 have a similar effect on lens cell differentiation. These studies suggest a novel gap-junction-independent role for Cx45.6 in lens epithelial-fiber differentiation.

### The cytoplasmic C-terminal domain is a determinant for the role of Cx45.6 in lens cell differentiation

The most diverse sequences among the various connexins lie in their cytoplasmic moieties, especially their long C-terminal domains (Goodenough et al., 1996). This domain does not directly participate in the formation of gap junction channels but is known to play divergent regulatory roles among different connexins. To determine whether this domain is involved in the unique role of Cx45.6 in lens cell differentiation, we generated chimeric connexin constructs in which the C-terminus of Cx45.6 was replaced with that of Cx56 (termed Cx45.6*56C), another lens fiber connexin, and in which the C-terminus of Cx56 was replaced with that of Cx45.6 (termed Cx56*45.6C) (Fig. 6A). We used recombinant retroviruses containing Cx45.6 chimeras along with wild-type Cx45.6 and Cx56 to infect lens primary cultures. Alongside β-actin controls, western blots with the antibody against the FLAG epitope showed similar levels of expression of Cx45.6 and recombinant constructs in primary lens cultures (Fig. 6B). Furthermore, scrape-loading dye-transfer analysis on CEF cells infected with these C-termini chimeras demonstrated that they formed functional gap junctions at a level comparable to that of their corresponding wild-type connexins (Fig. 6C). To determine the extent of lens cell differentiation with these constructs, the numbers of lentoids (Fig. 6D) and the expression of the differentiation marker MIP(AQP0) (Fig. 6E) were examined. As discussed above, lens cell differentiation is directly associated with lentoid formation in lens primary cultures. The total number of lentoids formed in the vehicle-treated culture or cultures expressing exogenous connexins was counted and quantified at various culture periods (Fig. 6D). The initiation and induction of lentoid bodies occurred at approximately 4 days in lens primary cultures. At the various times post infection tested, the total number of lentoids increased close to 100% with overexpression of wild-type Cx45.6 and Cx56*45.6C in comparison with Cx45.6*56C and vehicle controls. Notably, the stimulatory effect of Cx56*45.6C overexpression was almost identical to that of wild-type Cx45.6, whereas this effect was absent with the overexpression of Cx45.6*56C. Similar effects were noted for the levels of MIP(AQP0) expression (Fig. 6E).

To further investigate the role of the Cx45.6 C-terminus in lens differentiation, recombinant retroviruses containing the Cx45.6 C-terminus alone (termed: Cx45.6C), and Cx45.6 lacking its C-terminus [termed: Cx45.6(–C)] (Fig. 7A), were infected into lens primary cultures. The expression of these constructs was detected by western blot (Fig. 7B). Overexpression of Cx45.6C and Cx45.6(–C) failed to display any discernable effect on lentoid number in comparison with the vehicle control, RCAS(A) (Fig. 7C). The absence of an effect of the soluble C-terminus implies that only the membrane-attached C-terminus appears to affect lens differentiation, perhaps because the soluble C-terminus might not be in the identical conformation and/or correct intracellular localization in comparison with the wild-type Cx45.6 molecule. Together, these results suggest that the C-terminal domain, and not other Cx45.6 domains, is functionally involved in the process of lens cell differentiation.

## Discussion

In this study, we overexpressed wild-type Cx45.6 and Cx45.6 mutant proteins that cannot form gap junctions in lens primary

culture by using a recombinant retroviral expression system. The retroviral approach we used is highly effective; after retroviral infection, almost all the primary cultured cells expressed exogenous connexins (Jiang, 2001; Gu et al., 2003). We showed that expression of loss-of-function Cx45.6 mutants retained a capability similar to that of wild-type Cx45.6 in promoting lens epithelial-fiber cell differentiation. Importantly, even the dominant-negative mutants that blocked gap junction channels formed by native Cx45.6 were able to stimulate lens cell differentiation to a degree similar to that of the wild-type connexin. Furthermore, we identified the C-terminus of Cx45.6 as being an essential determinant for this specific function. This study, for the first time, demonstrates a gap-junction-independent role for a connexin in lens epithelial-fiber cell differentiation.

We observed that the stimulation of lens cell differentiation by Cx45.6 is independent of its conventional role as a component in the formation of functional gap junction channels. A similar stimulatory effect was also observed when overexpressing Cx45.6 constructs without the FLAG epitope tag (data not shown). The amino acid mutations examined (D47A and P88S) abolish the ability of Cx45.6 to form functional gap junction channels. However, these mutant proteins do not affect the normal expression and integrity of the Cx45.6 protein. Our previous studies also show that overexpression of Cx45.6 has no effect on endogenous Cx56 expression (Gu et al., 2003). Similar to wild-type Cx45.6, these mutant proteins are primarily distributed to the lentoid structures in primary lens cells. These observations suggest that these amino acid residues are important for formation of functional gap junction channels; however, these residues are unlikely to be involved in protein trafficking or expression. Importantly, we show that both mutations promote lens epithelial-fiber cell differentiation in a fashion almost identical to that of wild-type Cx45.6, which strongly favors a novel role for Cx45.6 in lens cell differentiation, independent of its role

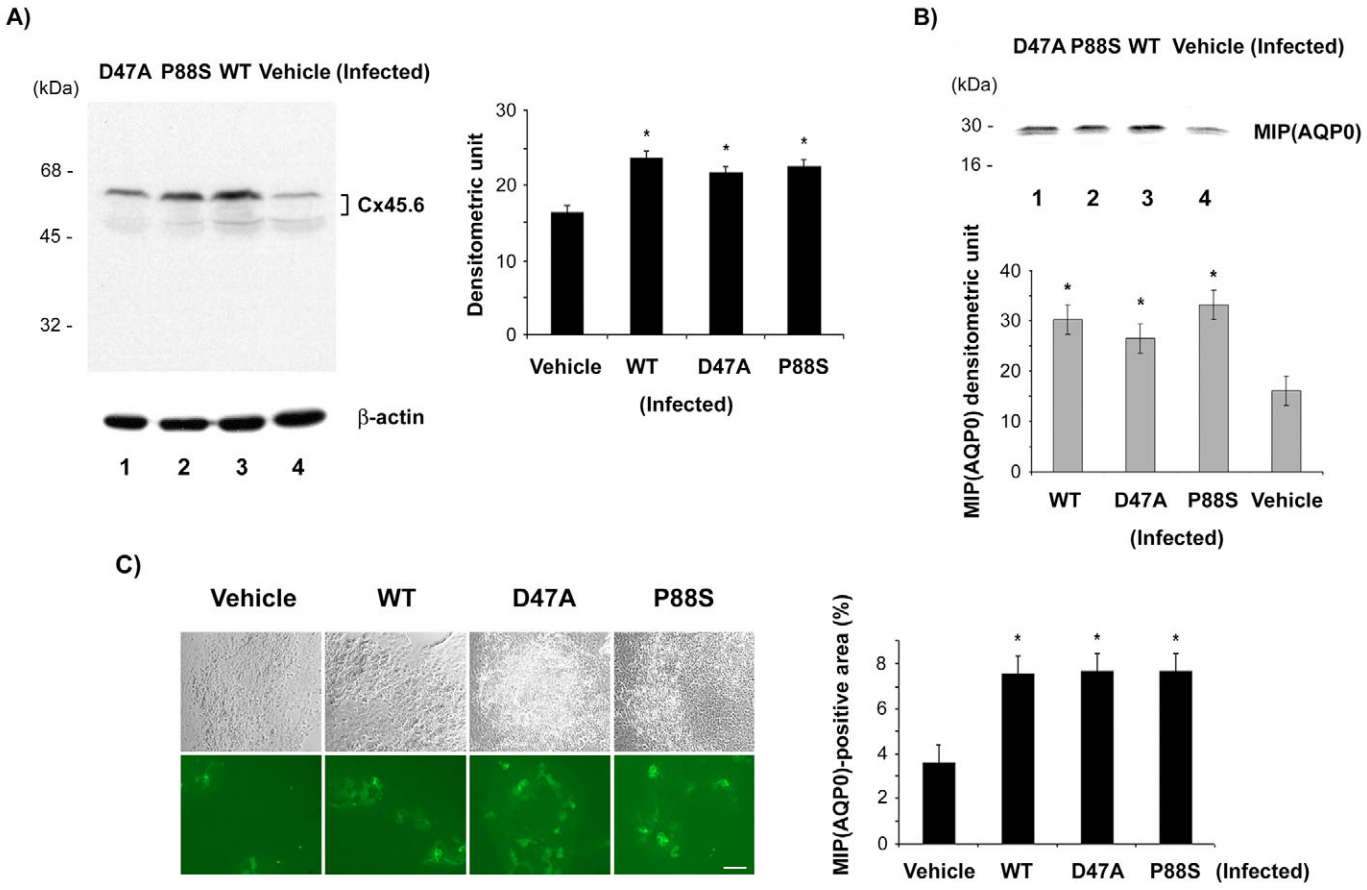


**Fig. 4.** Loss-of-gap-junction-function Cx45.6 mutants stimulate the expression of MIP(AQP0) to levels comparable to those of wild-type Cx45.6. Lens primary cultures were infected with recombinant retroviruses RCAS(A), RCAS(A)-Cx45.6, RCAS(A)-Cx45.6(D47A) or RCAS(A)-Cx45.6(P88S) for 8 days. (A) Crude membrane preparation from cells infected with retroviruses RCAS(A)-Cx45.6(D47A) (lane 1), RCAS(A)-Cx45.6(P88S) (lane 2), RCAS(A)-Cx45.6-WT (lane 3) and RCAS(A) vehicle (lane 4) were loaded on SDS-PAGE gels and immunoblotted with antibodies against Cx45.6, β-actin (A) or MIP(AQP0) (B). The Cx45.6 (A, right panel) and MIP(AQP0) (B, lower panel) bands from three separate western blot analyses were quantified by densitometry. The data are presented as the mean±s.e.m.; $n$=3. *$P$<0.05, in comparison with the non-Cx45.6-overexpressing control. (C) Primary cells were immunolabeled with monoclonal antibody against MIP(AQP0). The primary antibody was detected by fluorescein-conjugated anti-mouse IgG. The phase-contrast (upper panel) and immunostaining (lower panel) images were captured by a fluorescence microscope. The size of the MIP(AQP0)-stained area was quantified (UTHSCSA ImageTool Software) and presented as a percentage in the $x$-axis (C, right panel). The data are presented as the mean±s.e.m.; $n$=3. *$P$<0.05, in comparison with the non-Cx45.6-overexpressing control. Bar, 10 μm.

in forming gap junction channels. These data are in accord with our earlier observation that cell intercellular coupling is not altered by overexpression of wild-type Cx45.6 in primary lens cells (Gu et al., 2003). One potential explanation could be that the gap junction channels formed by endogenous Cx45.6 are sufficient for lens cell differentiation and the process is not affected by overexpression. However, our observations of comparable stimulation of lens cell differentiation by dominant-negative mutants render this possibility unlikely. To validate the dye-transfer analyses, we used two dye-transfer approaches: invasive scrape-loading dye transfer and non-invasive parachuting dye transfer. In addition, we used three types of tracer molecules: LY, Alexa 488 and calcein. We and others have shown that scrape-loading dye transfer for qualitatively assessing gap junction communication is consistent with microinjection results (Opsahl and Rivedal, 2000; Gu et al., 2003). One potential concern of scrape loading is the unknown effect of disrupting cell membrane permeability away from the scrape site. We therefore used a non-injurious cell parachuting technique to verify the scrape-loading dye-transfer conclusions. In our study, rhodamine dextran, employed as the non-transfering control for both Alexa 488 and LY, showed approximately identical travel distance from the scrape in all experiments. Therefore, the observed differences in dye-transfer distance between Alexa 488 and LY are most likely due to the selectivity properties of channels formed by Cx45.6. In fact, our observations confirm previous studies suggesting that gap junctions use selectivity filters that can somehow distinguish both size and charge (Nicholson et al., 2000; Weber et al., 2004).

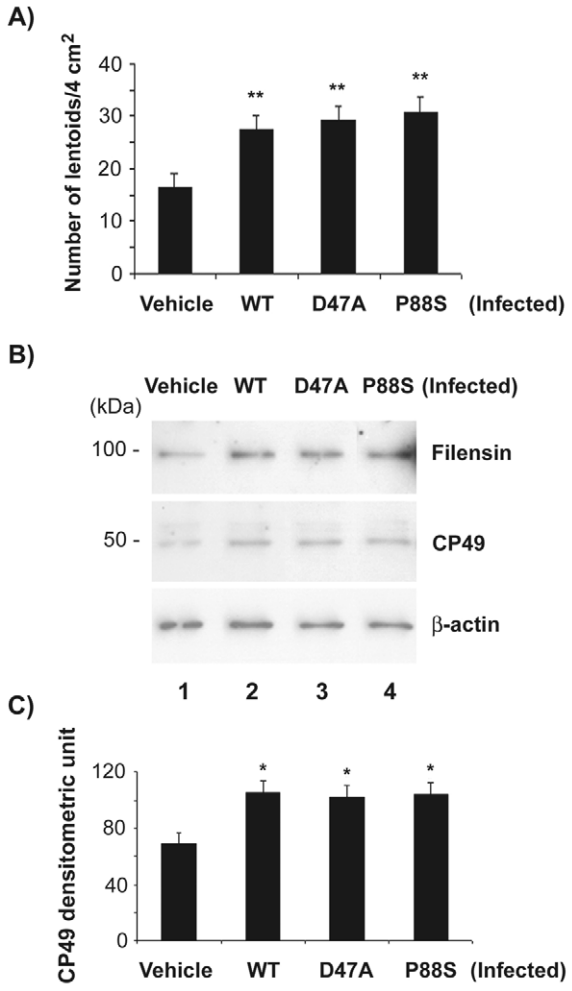


**Fig. 5.** Stimulation of lentoid formation and levels of lens differentiation markers filensin and CP49 by wild-type and loss-of-gap-junction mutants of Cx45.6. (A) Lens primary cultures were infected with recombinant retroviruses RCAS(A), RCAS(A)-Cx45.6, RCAS(A)-Cx45.6(D47A) or RCAS(A)-Cx45.6(P88S) for 8 days and the total numbers of lentoids were quantified. (B) Lysates from cells infected with retroviruses RCAS(A) vehicle (lane 1), RCAS(A)-Cx45.6-WT (lane 2), RCAS(A)-Cx45.6(D47A) (lane 3), and RCAS(A)-Cx45.6(P88S) (lane 4) were loaded on SDS-PAGE gels and immunoblotted with antibodies against filensin, CP49 or β-actin. (C) The CP49 protein bands from three separate western blot analyses were quantified by densitometry. **P*<0.05, in comparison with the non-Cx45.6-overexpressing control.

Along with other factors, expression of Cx45.6, but not the existence of active gap junctions, appears to be crucial for lens cell differentiation. Again, in accord with our observation, studies by Le and Musil (Le and Musil, 1998) show that the inhibition of intercellular couplings with the gap junction blocker 18β-glycyrrhetinic acid has no effect on lens epithelial-fiber differentiation and lentoid formation in primary lens cells. However, that study does not specify whether the process of lens cell differentiation requires connexin expression at all. It has been demonstrated that formation of gap junctions in lens primary culture is inhibited by exogenous expression of Src, which (1) does not inhibit synthesis of major junctional proteins; (2) interrupts incorporation of MP28 (analogous to chick MIP) into the plasma membrane, and (3) inhibits differentiation of lens cells (Menko and Boettiger, 1988). Although that study also did not address the contribution of connexins to the differentiation process, it did imply the importance of properly expressed gap junction proteins on the plasma membrane. Taken together, a number of studies support our hypothesis that Cx45.6 is unique among the lens connexins in possessing an extra-junctional role. There are several reports indicating gap-junction-independent roles for connexins in the control of cell growth and the suppression of tumorigenicity (Mesnil et al., 1995; Huang et al., 1998; Duflot-Dancer et al., 1997; Omori and Yamasaki, 1998; Olbina and Eckhart, 2003; Jiang and Gu, 2005). In two reports, the C-terminal domain of Cx43 alone is sufficient to suppress cell growth (Moorby and Patel, 2001; Dang et al., 2003).

In addition to being a component of gap junctions, connexin molecules can form hemichannels, which are non-apposed halves of the gap junction channels (Goodenough and Paul, 2003). A few studies have also shown that Cx50, the mammalian ortholog of chick Cx45.6, forms hemichannels in *Xenopus* oocytes and transfected HeLa cells (Beahm and Hall, 2002; Valiunas and Weingart, 2000), and hemichannels formed by lens connexins are voltage sensitive and possibly mechano-sensitive (Valiunas and Weingart, 2000; Bao et al., 2005). To determine the potential involvement of hemichannels, we expressed wild-type as well as D47A and P88S Cx45.6 mutant proteins in CEF cells by retroviral infection. In the absence of $Ca^{2+}$, the condition known to induce the opening of some hemichannels, we did not observe Cx45.6 hemichannel opening as compared with vehicle-treated controls (supplementary material Fig. S1). The difference between our

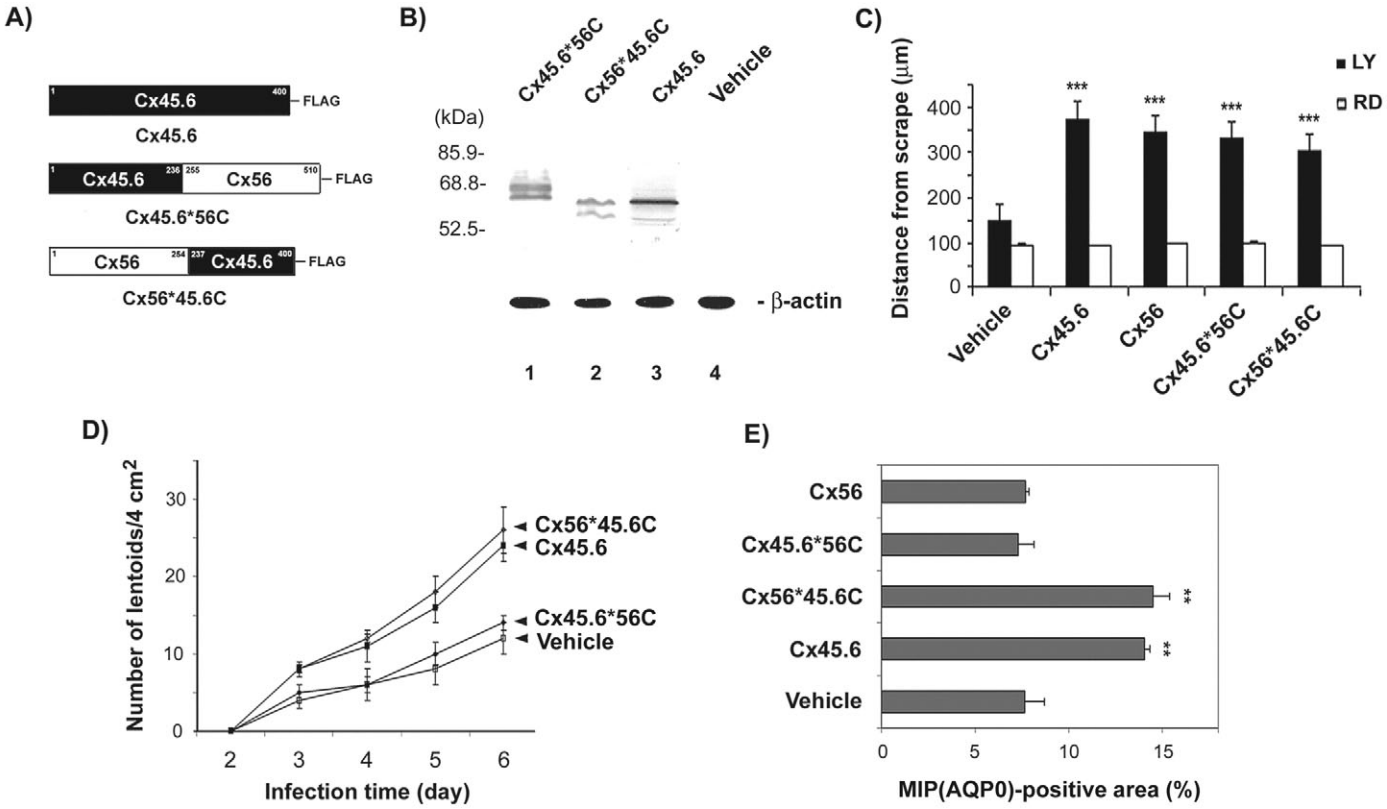


**Fig. 6.** Stimulation of lens differentiation by Cx56*45.6C but not by Cx45.6*56C. (A) Cx45.6*56C was generated by fusing Cx45.6 lacking its C-terminus with the C-terminus from Cx56. Cx56*45.6C was made by fusing Cx56 lacking its C-terminus with the C-terminus from Cx45.6. (B) Recombinant retroviruses containing cDNAs encoding wild-type Cx45.6 (lane 3), Cx45.6*56C (lane 1) and Cx56*45.6C (lane 2) or RCAS(A) vehicle control (lane 4) were infected into primary lens cultures. After 6 days of infection, lens cell lysates were analyzed by SDS-PAGE and immunoblotted with antibodies against FLAG or β-actin. (C) RCAS(A) retrovirus containing Cx45.6, Cx56, Cx45*56C and Cx56*45.6C sequences were infected into CEF cells and intercellular coupling was determined by scrape-loading dye transfer using LY-RD; $n$=3. (D) 6 days after infection, the total lentoid numbers were quantified at various time points; $n$=3. (E) 6 days after injection, primary lens cells were labeled with antibody against MIP and detected by fluorescein-conjugated anti-mouse IgG. The percentage of areas with MIP expression versus whole-image areas was determined; $n$=5. The data are presented as the mean±s.e.m.

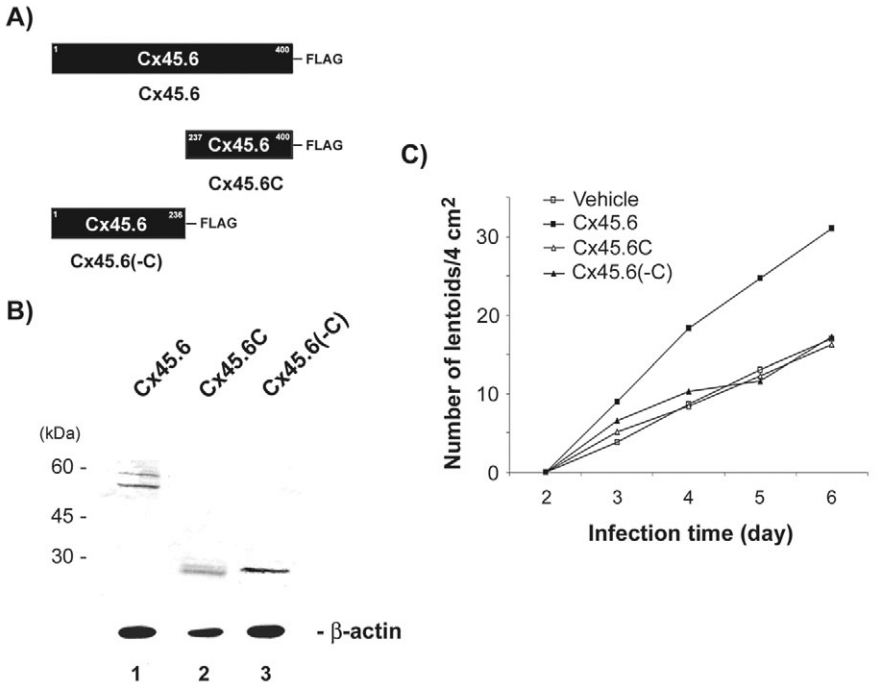


**Fig. 7.** Stimulation of lens differentiation by the C-terminus of Cx45.6. (A) Cx45.6C contains the entire C-terminus of Cx45.6. Cx45.6(–C) is a truncated mutant without its C-terminus. (B) Recombinant retroviruses containing wild-type Cx45.6 (lane 1), Cx45.6C (lane 2) and Cx45.6(–C) (lane 3) were infected into primary lens cultures. After 6 days of infection, lens cell lysates were analyzed by SDS-PAGE and immunoblotted with antibodies against FLAG or β-actin. (C) 6 days after infection, the total numbers of lentoids formed were quantified at various time points.

data and data concerning Cx50 hemichannel opening by electrophysiological methods could be due to the fact that these hemichannel openings are responsive to the applied voltage. As we identified that a non-pore-forming C-terminus, but not other domains of Cx45.6, is a determining domain in lens cell differentiation, it is unlikely that Cx45.6 hemichannels are involved in this differentiation process.

We show here that the chimeric connexin Cx56*45.6C, by replacing C-terminus of Cx56 with that of Cx45.6, is sufficient to promote lens cell differentiation to an extent similar to that of wild-type Cx45.6. However, the soluble C-terminal domain has no such stimulatory effect, which could be caused by the lack of membrane localization and/or an inappropriate protein conformation. Endogenous Cx45.6 and Cx56 colocalize throughout the embryonic lenses and form heteromeric connexons in the lens (Jiang and Goodenough, 1996). Despite their identical localization and coexistence in the same connexons, we have identified that expression of Cx45.6 promotes lens cell differentiation. Previous reports have shown that the orthologs of Cx45.6 and Cx56 display differential physiological and gating properties: the Cx45.6 ortholog displays a high sensitivity in pH gating and appears to regulate the Cx56 ortholog in differentiating fibers (Lin et al., 1998; Baldo et al., 2001; Eckert, 2002), whereas the Cx56 ortholog exhibits less pH sensitivity and is responsible for coupling mature fibers (Eckert, 2002; Baldo et al., 2001). These physiological differences could be accounted for partially by the uniqueness of the C-terminal sequences possessed by different 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. A direct interaction between the C-terminal domains of gap junction proteins Cx43 and Cx45 with ZO-1 has been observed; this association might serve to localize connexins to certain regions in cells (Toyofuku et al., 1998; Laing et al., 2001). Recent findings suggest that connexins interact with other proteins, including occludin, claudins, N-cadherin and the cytoskeletal proteins forming microtubules, actin and catenins (Giepmans et al., 2001; Xu et al., 2001; Wu et al., 2003; Theiss and Meller, 2002; Duffy et al., 2002; El-Sabban et al., 2003). The potential interactions of the Cx45.6 C-terminal domain with other factor(s) that might facilitate its role in lens fiber differentiation and formation will be the subject of further investigation.

## Materials and Methods

### Materials

Fertilized, unincubated white leghorn chicken eggs were obtained from Ideal Poultry (Cameron, TX) and incubated for 11 days in a humidified 37°C incubator. Anti-Cx45.6 antibodies were raised and affinity purified as previously reported (Jiang et al., 1994). Anti-chick MIP(AQP0) monoclonal antibody was a generous gift from Erica Tenbroek and Ross Johnson from the University of Minnesota (St Paul, MN), and anti-filensin and CP49 antibodies were generous gifts from Paul FitzGerald from the University of California (Davis, CA). Anti-MIP(AQP0) polyclonal antibody was purchased from Alpha Diagnostics (San Antonio, TX); rhodamine-conjugated goat anti-mouse IgG and bicinchoninic acid (BCA) microprotein assay kit from Pierce Chemical (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); PVDF membrane from Bio-Rad (Hercules, CA); fetal bovine serum (FBS) from Hyclone Laboratories (Logan, UT); QuikChange™ site-directed mutagenesis kit from Stratagene (La Jolla, CA); Vectashield® fluorescence mounting medium from Vector Laboratories (Burlingame, CA); chemiluminescence kit (ECL) from Amersham Pharmacia Biotech (Piscataway, NJ); Alexa 488, Lucifer yellow (LY), rhodamine dextran, calcein acetoxymethyl ester (AM), 4′,6-diamidino-2-phenylindole (DAPI) and dialkylcarbocyanine dye (DiI) 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 encoding Cx45.6 and Cx45.6 mutant proteins and generation of high-titer retroviruses

Retroviral constructs and high-titer retroviruses were prepared based on our protocol described previously (Jiang, 2001). In brief, a cDNA fragment containing wild-type Cx45.6 was made by PCR and was constructed into the retroviral vector RCAS(A) as described previously (Jiang and Goodenough, 1998; Yin et al., 2000). With the wild-type RCAS(A)-Cx45.6 DNA construct as a template, retroviral constructs of Cx45.6 mutants containing point mutations were generated with the QuikChange™ site-directed mutagenesis kit according to the manufacturer's instructions with the following pairs of primers: Cx45.6(D47A) (sense: CTAGTATGGGGAGCT-GAACAGTCAGAC, antisense: GTCTGACTGTTCAGCTCCCCATACTAG); Cx45.6(P88S) (sense: CATTTTTGTATCCACGTCTTCGCTAGTGTACTTTGGG, antisense: CCCAAAGTACACTAGCGAAGACGTGGATACAAAAATG). RCAS(A)-Cx45.6(D47A) was made by changing codon GAT encoding Asp to GCT encoding Ala and RCAS(A)-Cx45.6(P88S) by changing codon CCT encoding Pro to TCT encoding Ser. The C-termini of both wild-type and mutant Cx45.6 were epitope tagged with FLAG sequences to distinguish the exogenous from endogenous connexins. For the construction of chimeric retrovirus Cx45.6*56C, a two-step PCR procedure was carried out. By using RCAS(A)-Cx45.6 retrovirus as a template, we first obtained a fragment containing C-terminally truncated Cx45.6 (amino acids 1-236) as well as the retroviral vector RCAS(A) with the following pair of primers: sense: TACGAATTCGAGCTCGCCCGGGGA, antisense: CCTCCGGATCCTTT-TCAGGAT. Next, by using RCAS(A)-Cx56 as a template, a fragment containing the C-terminus from Cx56 (amino acids 255-510) was obtained with the following pair of primers: sense: GGCATGACAAGCCAGTACAGC, antisense: GCGAATT-CTTACTTGTCATCGTCGTCCTTGTAGTCCACTGCCAAGTCATC. Finally, the two fragments were ligated together to form the complete Cx45.6*56C construct. Similarly, for chimeric retrovirus Cx56*45.6C, two fragments were generated. The first fragment contained the C-terminus truncated Cx56 (amino acids 1-254) and RCAS(A) vector by using RCAS(A)-Cx56 as a template and the following pair of primers: sense: TACGAATTCGAGCTCGCCCGGGGA, antisense: CTGCTTAA-GCTTCTTCCATCC. The second fragment contained the C-terminus of Cx45.6 (amino acids 237-400) by using RCAS(A)-Cx45.6 as a template and the following pair of primers: sense: GCTCTGAGAAGACCAGCAGAG, antisense: GCGAA-TTCTTACTTGTCATCGTCGTCCTTGTAGTCTACAGTCAGATCGTC. The two fragments were then fused together to form the complete Cx56*45.6C construct. For retrovirus containing only the C-terminal truncated form of Cx45.6, RCAS(A)-Cx45.6 cDNA was treated with two restriction enzymes *Bgl*II and *Bst*EII to remove the C-terminal regions of Cx45.6, and the rest of the fragment (amino acids 1-236) was ligated to form the Cx45.6C retroviral construct. PCR primers required for generation of these mutants were synthesized at the University of Texas Health Science Center (UTHSCSA) DNA Core Facility. All the constructs generated were also sequenced at the UTHSCSA DNA Core Facility. High-titer recombinant retroviruses were then amplified through several passages of chicken embryonic fibroblast (CEF) cultures. High-titer retroviruses containing wild-type and mutant Cx45.6 cDNA were generated ($8\times10^8$ to $10\times10^8$ cfu/ml).

### Primary lens cell culture

Primary lens cell cultures were prepared by a modified method as described previously (Menko et al., 1984). Lenses from 10-11-day-old chick embryos were dissected, washed with TD buffer [140 mM NaCl, 5 mM KCl, 0.7 mM $Na_2HPO_4$, 5 mM glucose and 25 mM Tris (pH 7.4)], and digested with 0.1% trypsin in TD buffer at 37°C, and then broken apart by pipetting up and down in M199 media (plus 10% FBS and 1% penicillin/streptomycin). Cells were collected and resuspended in M199 media. Living cells were then counted and seeded at $3\times10^5$ cells per well of 12-well culture plates. The next day after the primary culture was seeded, retroviruses containing wild-type and mutant cDNA of Cx45.6 diluted in M199 were added to primary lens cultures for overexpression of connexins. The cultures were incubated at 37°C, 5% $CO_2$ and fed every other day. At the start of culturing, only monolayer lens epithelial cells proliferated on the culture plates, but not fiber cells. After 3-4 days, lens epithelial cells became confluent and began to differentiate and form fiber-like 'lentoid' structures.

### Immunofluorescence and confocal laser microscopy

For immunolabeling of connexins expressed in the primary lens culture, a glass cover slip was placed into each well of a 12-well plate before cell seeding. After approximate 6-8 days of culturing, lens epithelial cells were substantially differentiated into lens fiber cells, associated with the formation of lentoid structures. Cells were fixed by 2% PFA for 30 minutes and then incubated with blocking solution (2% goat serum, 2% fish skin gelatin, 0.25% Triton-X-100 and 1% BSA in Hank's balanced salt solution (HBSS) for another 30 minutes.

Exogenous connexins were labeled using anti-FLAG primary antibody (1:400 dilution) followed by FITC-conjugated anti-mouse IgG (1:500 dilution). Total connexins were labeled using Cx45.6 primary antibody (1:400 dilution) followed by rhodamine-conjugated anti-rabbit antibody (1:500 dilution). The nucleus was counterstained with DAPI (1:5000 dilution). For measurement of MIP(AQP0) expression, anti-chick MIP(AQP0) monoclonal antibody (Hybridoma supernatant, 1:1 dilution) was used followed by FITC-conjugated anti-mouse secondary antibody (1:500 dilution). Finally, the glass slip was laid on a microscopic slide and covered with a drop of mounting medium and sealed with nail polish. The specimens were analyzed using a confocal laser scanning microscope (Fluoview; Olympus Optical, Tokyo, Japan). Acquisition conditions were kept constant for each sample. FITC fluorescence was excited at 488 nm by an argon laser with corresponding excitation wavelengths from 505-525 nm using a barrier filter. Rhodamine was excited at 543 nm with a HeNe-G laser with a corresponding excitation wavelength of 560 nm using a long-pass barrier filter. DAPI fluorescence was excited at 405 nm with corresponding excitation wavelengths from 430-460 nm using a barrier filter. For visual MIP quantitation, we used a method previously developed in our laboratory. Primary cultures grown on plastic and glass coverslips in 12-well culture dishes were used for MIP(AQP0) quantification. Representative fluorescent images from various regions within each culture dish were used to determine the MIP staining area versus the whole image area (Gu et al., 2003). The threshold was adjusted to clearly distinguish lentoid boundaries for each image used for lentoid measurement. Fluorescent images were then converted to a binary black-white scale where black represented the original FITC fluorescence. Lastly the black pixels were converted to a percentage of the total black-white field and reported as MIP-positive area using the UTHSCSA ImageTool Image Analysis Software. Ten to 15 random images for each condition tested were used to assess MIP expression per measurement.

### Scrape-loading dye-transfer assay and fluorescence microscopy

CEF cells were grown to confluence to maximize cell-cell contact. The CEF cell morphology makes it difficult to visualize dye transfer through individual cells; therefore, a total distance of dye transfer from scrape loading was used. Scrape-loading dye transfer was performed based on a modified procedure (El-Fouly et al., 1987). Briefly, cells were scratched in the presence of two fluorescent dyes: Alexa 488 (molecular mass 570 Da) or Lucifer yellow (LY; 457 Da) that can pass through gap junction channels, plus rhodamine dextran (RD; 10 kDa), which is too large to pass through gap junction channels. Therefore, the presence of Alexa 488/LY indicates cells participating in gap junction-mediated communication and RD serves as a tracer dye for cells originally receiving the dyes. Cells were washed three times with HBSS plus 1% BSA for 5 minutes each, and then a mix containing 1 mM Alexa 488/1% RD or 1% LY/1% RD in PBS was applied, after which plates were scraped lightly with a 261/2-gauge needle. After 15 minutes incubation, cells were washed with HBSS three times, twice with PBS and then fixed in fresh 2% paraformaldehyde for 30 minutes. Dye-transfer results were examined using a fluorescence microscope (Olympus) in which Alexa 488/LY and RD could be detected by using fluorescein and RD filters, respectively. Acquisition conditions were kept consistent for all measurements and no threshold adjustments were used. Using RD staining as the reference for original dye-loaded cells, the extent of dye transfer was measured as the distance from the RD-labeled scrape line to the farthest extent of Alexa 488/LY-stained cells with the appropriate scale bar. At least five images per condition tested with six measurements per image were used to assess the extent of dye transfer.

### Cell parachuting dye-transfer assay

This approach was based on a published protocol (Goldberg et al., 1995) with some modifications. Briefly, CEF cells expressing exogenous wild-type and mutant Cx45.6 owing to retroviral infection were grown to confluence in 60 mm and 100 mm culture plates. Cells infected with retrovirus containing the RCAS(A) vector without connexins was used as a control (vehicle). The donor cells cultured in 60 mm plates were incubated with calcein AM and DiI for 20 minutes at room temperature. Gap junction intercellular communication can be followed by simultaneously labeling cells with calcein AM as a cytosolic tracer dye along with the membrane label DiI (Goldberg et al., 1995). Once inside the cell, calcein AM is cleaved by non-specific esterases into calcein (994.87 Da), which then fluoresces in a manner similar to LY and Alexa 488. DiI fluoresces in a manner similar to rhodamine dextran and indicates cell boundaries of preloaded cells. Preloaded cells from 60 mm plates were then resuspended in 5 ml culture media from which 200 μl were aliquoted and layered ('parachuted') over the top of the respective unlabeled receptor cells cultured in 100 mm plates. Cells were allowed to attach for 4 hours and examined by fluorescence microscope. Images were analyzed similarly as previously described for the MIP-positive area measurements. For calcein dye transfer, the threshold was adjusted to distinguish clearly dye-transfer boundaries for each image used for measurement. Images were again converted to a binary black-white scale, with black representing the original calcein fluorescence. Finally, the black pixels were converted to a percentage of the total black-white field and reported as calcein-positive area. Five representative images for each condition tested were used to assess calcein dye transfer per measurement.

### Dye-uptake assay

The dye-uptake analysis was performed as previously described (Cherian et al., 2005). Briefly, CEF cells were grown at low cell density to ensure that the majority of the cells were not physically in contact. LY was used as a tracer for hemichannel activity, and RD was used as a negative control. Cells maintained in $Ca^{2+}$-free MEM were exposed to 5 mM EGTA for 15 minutes. After treatment, dye-uptake experiments were conducted in the presence of 0.4% LY and 0.4% RD for 5 minutes, and the cells were washed with medium containing 1.8 mM $Ca^{2+}$ and then fixed with 1% paraformaldehyde.

### SDS gel electrophoresis, fluorography and western blot

Briefly, cultured cells were collected in lysis buffer (5 mM Tris pH 8.0 and 5 mM EDTA/EGTA) and then ruptured by pipetting through a 261/2 gauge needle. Lysates were then spun for 3 minutes at 1000 ***g*** to remove cell debris. The supernatant was then spun at100,000 ***g*** for 30 minutes (SW 60 Ti rotor, Beckman). The pellet was then resuspended in lysis buffer and boiled in 0.6% SDS for 3 minutes. Lysates were analyzed by 12% SDS-PAGE. Western blots were performed by probing with anti-MIP(AQP0) (1:300 dilution), anti-filensin (1:1000 dilution), anti-CP49 (1:1000 dilution) affinity-purified anti-Cx45.6 (1:500 dilution) and anti-β-actin (1:5000 dilution). Primary antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000 dilution) or anti-mouse IgG (1:5000 dilution) using chemiluminescence reagent kit (ECL). The intensity of the bands on western blots was quantified by densitometry.

### Statistical analysis

Data were analyzed with one-way ANOVA and Newman-Keuls multiple comparison test along with a biostatistics program (Prism). Data are presented as the mean±s.e.m. of a least three measurements. Asterisks represent the degree of significance in comparison with controls (*$P$<0.05; **$P$<0.01; ***$P$<0.001).

The work was supported by grants from the NIH (EY12085 to J.X.J.) and the Welch Foundation (AQ-1507 to J.X.J.). We thank Ross Johnson and Erica Tenbroek for generously providing the antibody against MIP, and Paul FitzGerald for antibodies against filensin and CP49. Confocal images were generated in the Core Optical Imaging Facility, which is supported by UTHSCSA, NIH-NCI P30 CA54174 (San Antonio Cancer Institute), NIH-NIA P30 AG013319 (Nathan Shock Center) and (NIH-NIA P01AG19316), and we thank the staff for their assistance. Finally, we thank Sumin Gu for technical assistance and members of J.X.J.'s laboratory for critical reading of the manuscript.

## References

**Baldo, G., Gong, X., Martinez-Wittinghan, F. J., Kumar, N., Gilula, N. B. and Mathias, R. T.** (2001). Gap junctional coupling in lenses from $\alpha_8$ connexin knockout mice. *J. Gen. Physiol.* **118**, 447-456.

**Bao, L., Sachs, F. and Dahl, G.** (2005). Connexins are mechanosensitive. *Am. J. Physiol. Cell Physiol.* **287**, C1389-C1395.

**Beahm, D. L. and Hall, J. E.** (2002). Hemichannel and junctional properties of connexin 50. *Biophys. J.* **82**, 2016-2031.

**Berry, V., Mackay, D., Khaliq, S., Francis, P. J., Hameed, A., Anwar, K., Mehdi, S. Q., Newbold, R. J., Ionides, A., Shiels, A. et al.** (1999). Connexin 50 mutation in a family with congenital "zonular nuclear" pulverulent cataract of Pakistani origin. *Hum. Genet.* **105**, 168-170.

**Berthoud, V. M., Bassnett, S. and Beyer, E. C.** (1999). Cultured chicken embryo lens cells resemble differentiating fiber cells in vivo and contain two kinetic pools of connexin56. *Exp. Eye Res.* **68**, 475-484.

**Blankenship, T. N., Hess, J. F. and FitzGerald, P. G.** (2001). Development- and differentiation-dependent reorganization of intermediate filaments in fiber cells. *Invest. Opthalmol. Vis. Sci.* **42**, 735-742.

**Bloemendal, H.** (1977). The vertebrate eye lens. *Science* **197**, 127-138.

**Cherian, P. P., Siller-Jackson, A. J., Gu, S., Wang, X., Bonewald, L. F., Sprague, E. and Jiang, J. X.** (2005). Hemichannels formed by connexin 43 provide a novel pathway for the release of prostaglandin E2 by osteocytes in response to mechanical strain. *Mol. Biol. Cell* **16**, 3100-3106.

**Dahm, R.** (1999). Lens fibre cell differentiation – a link with apoptosis? *Ophthalmic Res.* **31**, 163-183.

**Dang, X. T., Doble, B. W. and Kardami, E.** (2003). The carboxy-tail of connexin-43 localizes to the nucleus and inhibits cell growth. *Mol. Cell. Biochem.* **242**, 35-38.

**Duffy, H. S., Delmar, M. and Spray, D. C.** (2002). Formation of the gap junction nexus: binding partners for connexins. *J. Physiol. Paris* **96**, 243-249.

**Duflot-Dancer, A., Mesnil, M. and Yamasaki, H.** (1997). Dominant-negative abrogation of connexin-mediated cell growth control by mutant connexin genes. *Oncogene* **15**, 2151-2158.

**Eckert, R.** (2002). pH gating of lens fiber connexins. *Pflügers Arch.* **443**, 843-851.

**El-Fouly, M. H., Trosko, J. E. and Chang, C. C.** (1987). Scrape-loading and dye transfer. A rapid and simple technique to study gap junctional intercellular communication. *Exp. Cell Res.* **168**, 422-430.

**El-Sabban, M. E., Abi-Mosleh, L. F. and Talhouk, R. S.** (2003). Developmental regulation of gap junctions and their role in mammary epithelial cell differentiation. *J. Mammary Gland Biol. Neoplasia* **8**, 463-473.

**Gerido, D. A. and White, T. W.** (2004). Connexin disorders of the ear, skin, and lens. *Biochim. Biophys. Acta* **1662**, 159-170.

**Giepmans, B. N. G., Verlann, I., Hengeveld, T., Janssen, H., Calafat, J., Falk, M. M. and Moolenaar, W. H.** (2001). Gap junction protein connexin-43 interacts directly with microtubules. *Curr. Biol.* **11**, 1364-1368.

**Goldberg, G. S., Bechberger, J. F. and Naus, C. C. G.** (1995). A pre-loading method of evaluating gap junctional communication by fluorescent dye transfer. *Biotechniques* **18**, 490-497.

**Gong, X., Li, E., Klier, G., Huang, Q., Wu, Y., Lei, H., Kumar, N. M., Horwitz, J. and Gilua, N. B.** (1997). Disruption of $\alpha_3$ connexin gene leads to proteolysis and cataractogenesis in mice. *Cell* **91**, 833-843.

**Goodenough, D. A.** (1992). The crystalline lens: a system networked by gap junctional intercellular communication. *Semin. Cell Biol.* **3**, 49-58.

**Goodenough, D. A. and Paul, D. L.** (2003). Beyond the gap: functions of unpaired connexon channels. *Nat. Rev.* **4**, 1-10.

**Goodenough, D. A., Goliger, J. A. and Paul, D. L.** (1996). Connexins, connexons, and intercellular communication. *Annu. Rev. Biochem.* **65**, 475-502.

**Gu, S., Yu, X. S., Yin, X. and Jiang, J. X.** (2003). Stimulation of lens cell differentiation by gap junction protein connexin 45.6. *Invest. Ophthalmol. Vis. Sci.* **44**, 2103-2111.

**Huang, R. P., Fan, Y., Hossain, M. Z., Peng, A., Zeng, Z. L. and Boynton, A. L.** (1998). Reversion of the neoplastic phenotype of human glioblastoma cells by connexin43 (cx43). *Cancer Res.* **58**, 5089-5096.

**Jiang, J. X.** (2001). Use of retroviruses to express connexins. In *Connexin Methods and Protocols* (ed. R. Bruzzone and C. Giaume), pp. 159-174. Totowa: Humana Press.

**Jiang, J. X. and Goodenough, D. A.** (1996). Heteromeric connexons in lens gap junction channels. *Proc. Natl. Acad. Sci. USA* **93**, 1287-1291.

**Jiang, J. X. and Goodenough, D. A.** (1998). Retroviral expression of connexins in embryonic chick lens. *Invest. Ophthalmol. Vis. Sci.* **39**, 537-543.

**Jiang, J. X. and Gu, S.** (2005). Gap junction- and hemichannel-independent actions of connexins. *Biochim. Biophys. Acta* **1711**, 208-214.

**Jiang, J. X., Paul, D. L. and Goodenough, D. A.** (1993). Posttranslational phosphorylation of lens fiber connexin46: a slow occurrence. *Invest. Ophthalmol. Vis. Sci.* **34**, 3558-3565.

**Jiang, J. X., White, T. W., Goodenough, D. A. and Paul, D. L.** (1994). Molecular cloning and functional characterization of chick lens fiber connexin45.6. *Mol. Biol. Cell* **5**, 363-373.

**Jiang, J. X., White, T. W. and Goodenough, D. A.** (1995). Changes in connexin expression and distribution during chick lens development. *Dev. Biol.* **168**, 649-661.

**Laing, J. G., Manley-Markowski, R. N., Koval, M., Civitelli, R. and Steinberg, T. H.** (2001). Connexin45 interacts with zonula occludens-1 and connexin43 in osteoblastic cells. *J. Biol. Chem.* **276**, 23051-23055.

**Le, A.-C. N. and Musil, L. S.** (1998). Normal differentiation of cultured lens cells after inhibition of gap junction-mediated intercellular communication. *Dev. Biol.* **204**, 80-96.

**Lin, J. S., Eckert, R., Kistler, J. and Donaldson, P.** (1998). Spatial differences in gap junction gating in the lens are a consequence of connexin cleavage. *Eur. J. Cell Biol.* **76**, 246-250.

**Mackay, D., Ionides, A., Kibar, Z., Rouleau, G., Berry, V., Moore, A., Shiels, A. and Bhattacharya, S.** (1999). Connexin46 mutations in autosomal dominant congenital cataract. *Am. J. Hum. Genet.* **64**, 1357-1364.

**Martinez-Wittinghan, F. J., Sellitto, C., Li, L., Gong, X., Brink, P. R., Mathias, R. T. and White, T. W.** (2003). Dominant cataracts result from incongruous mixing of wild-type lens connexins. *J. Cell Biol.* **161**, 969-978.

**Mathias, R. T., Rae, J. L. and Baldo, G. J.** (1997). Physiological properties of the normal lens. *Physiol. Rev.* **77**, 21-50.

**McAvoy, J. W., Chamberlain, C. G., de Iongh, R. U., Hales, A. M. and Lovicu, F. J.** (1999). Lens development. *Eye* **13**, 425-437.

**Menko, A. S. and Boettiger, D.** (1988). Inhibition of chicken embryo lens differentiation and lens junction formation in culture by pp60v-src. *Mol. Cell. Biol.* **8**, 1414-1420.

**Menko, A. S., Klukas, K. A. and Johnson, R. G.** (1984). Chicken embryo lens cultures mimic differentiation in the lens. *Dev. Biol.* **103**, 129-141.

**Mesnil, M., Krutovskikh, V., Piccoli, C., Elfgang, C., Traub, O., Willecke, K. and Yamasaki, H.** (1995). Negative growth-control of Hela-cells by connexin genes-connexin species-specificity. *Cancer Res.* **55**, 629-639.

**Moorby, C. and Patel, M.** (2001). Dual functions for connexins: Cx43 regulates growth independently of gap junction formation. *Exp. Cell Res.* **271**, 238-248.

**Musil, L. S., Beyer, E. C. and Goodenough, D. A.** (1990). Expression of the gap junction protein connexin43 in embryonic chick lens: molecular cloning, ultrastructural localization, and post-translational phosphorylation. *J. Membr. Biol.* **116**, 163-175.

**Nicholson, B. J., Weber, P. A., Cao, F., Chang, H., Lampe, P. and Goldberg, G.** (2000). The molecular basis of selective permeability of connexins is complex and includes both size and charge. *Braz. J. Med. Biol. Res.* **33**, 369-378.

**Olbina, G. and Eckhart, W.** (2003). Mutations in the second extracellular region of connexin 43 prevent localization to the plasma membrane, but do not affect its ability to suppress cell growth. *Mol. Cancer Res.* **1**, 690-700.

**Omori, Y. and Yamasaki, H.** (1998). Mutated connexin43 proteins inhibit rat glioma cell growth suppression mediated by wild-type connexin43 in a dominant-negative manner. *Int. J. Cancer* **78**, 446-453.

**Opsahl, H. and Rivedal, E.** (2000). Quantitative determination of gap junction intercellular communication by scrape loading and image analysis. *Cell Adhes. Commun.* **7**, 367-375.

**Pal, J. D., Berthoud, V. M., Beyer, E. C., Mackay, D., Shiels, A. and Ebihara, L.** (1999). Molecular mechanism underlying a Cx50-linked congenital cataract. *Am. J. Physiol.* **276**, C1443-C1446.

**Rees, M. I., Watts, P., Fenton, I., Clarke, A., Snell, R. G., Owen, M. J. and Gray, J.** (2000). Further evidence of autosomal dominant congenital zonular pulverulent cataracts linked to 13q11 (CZP3) and a novel mutation in connexin 46 (GJA3). *Hum. Genet.* **106**, 206-209.

**Rong, P., Wang, X., Niesman, I., Wu, Y., Benedetti, L. E., Dunia, I., Levy, E. and Gong, X.** (2002). Disruption of *Gja8* ($\alpha$8 connexin) in mice leads to microphthalmia associated with retardation of lens growth and lens fiber maturation. *Development* **129**, 167-174.

**Rup, D. M., Veenstra, R. D., Wang, Z., Brink, P. R. and Beyer, E. C.** (1993). Chick connexin-56, a novel lens gap junction protein. *J. Biol. Chem.* **268**, 706-712.

**Shiels, A., Mackay, D., Ionides, A., Berry, V., Moore, A. and Bhattacharya, S.** (1998). A missense mutation in the human connexin50 gene (GJA8) underlies autosomal dominant "zonular pulverulent" cataract, on chromosome 1q. *Am. J. Hum. Genet.* **62**, 526-532.

**Steele, E. C., Jr, Lyon, M. F., Favor, J., Guillot, P. V., Boyd, Y. and Church, R. L.** (1998). A mutation in the connexin50 (Cx50) gene is a candidate for the No2 mouse cataract. *Curr. Eye Res.* **17**, 883-889.

**Theiss, C. and Meller, K.** (2002). Microinjected anti-actin antibodies decrease gap junctional intercellular communication in cultured astrocytes. *Exp. Cell Res.* **281**, 197-204.

**Toyofuku, Y., Yabuki, M., Otsu, K., Kuzuya, T., Hori, M. and Tada, M.** (1998). Intercellular calcium signaling via gap junction in connexin-43-transfected cells. *J. Biol. Chem.* **273**, 1519-1528.

**Valiunas, V. and Weingart, R.** (2000). Electrical properties of gap junction hemichannels identified in transfected cells. *Pflugers Arch.* **440**, 366-379.

**Weber, P. A., Chang, H. C., Spaeth, K. E., Nitsche, J. M. and Nicholson, B. J.** (2004). The permeability of gap junction channels to probes of different size is dependent on connexin composition and permeant-pore affinities. *Biophys. J.* **87**, 958-973.

**White, T. W.** (2002). Unique and redundant connexin contributions to lens development. *Science* **295**, 319-320.

**White, T. W., Goodenough, D. A. and Paul, D. L.** (1998). Targeted ablation of connexin50 in mice results in microphthalmia and zonular pulverulent cataracts. *J. Cell Biol.* **143**, 815-825.

**Wu, J. C., Tsai, R. Y. and Chung, T. H.** (2003). Role of catenins in the development of gap junctions in rat cardiomyocytes. *J. Cell. Biochem.* **88**, 823-835.

**Xu, X. and Ebihara, L.** (1999). Characterization of a mouse Cx50 mutation associated with the No2 mouse cataract. *Invest. Ophthalmol. Vis. Sci.* **40**, 1844-1850.

**Xu, X., Li, W. E. I., Huang, G. Y., Meyer, R., Chen, T., Luo, Y., Thomas, M. P., Radice, G. L. and Lo, C. W.** (2001). Modulation of mouse neural crest cell mobility by N-cadherin and connexin 43 gap junctions. *J. Cell Biol.* **154**, 217-229.

**Yin, X., Jedrzejewski, P. T. and Jiang, J. X.** (2000). Casein kinase II phosphorylates lens connexin 45.6 and is involved in its degradation. *J. Biol. Chem.* **275**, 6850-6856.

**Yu, X. S. and Jiang, J. X.** (2004). Interaction of major intrinsic protein (aquaporin-0) with fiber connexins in lens development. *J. Cell Sci.* **117**, 871-880.

**Zhou, L., Chen, T. and Church, R. L.** (2002). Temporal expression of three mouse lens fiber cell membrane protein genes during early development. *Mol. Vis.* **8**, 143-148.